GENETICAL AND CYTOLOGICAL STUDIES ON VARIATIONS OF *VOLVARIELLA VOLVACEA*

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ABSTRACT

Volvariella volvacea (Bull. ex Fr.) Sing., commonly known as the straw mushroom, is an important edible fungus of the tropics and subtropics. Although considerable variation of monosporous isolates in *V. volvacea* have been found for about twenty years, the mechanism of variation and the sexuality pattern of this species are still not fully understood.

In order to circumvent these problems, three studies were conducted. The purpose of the first study was to confirm the phenotype variations, and then two strategies, genetical and cytological approaches, were used to investigate the sexuality pattern and possible sources of variation in *V. volvacea*.

Results from 328 monosporous isolates derived from five geographical strains show that there was a wide range of variation in monosporous isolates even though they were isolated from one sporocarp. These variations occurred not only in growth rate but also in colonial morphology, fertility and extracellular cellulase activity.

For genetical study, attempts have been made to induce auxotrophic and resistant mutants with physical and chemical mutagenesis as well as to utilize an enrichment method for selection of auxotrophs. Although it is very difficult to induce mutants in *V. volvacea* due to the multinucleate nature of hyphal cells

and thick-walled spores, two kinds of stable and fertile mutants, malachite green resistant mutant and aspartate-requiring mutant, have been obtained. Over 90% of the progeny of the mutants retained their parental marker phenotype. It could be concluded that *V. volvacea* is a primary homothallic species. Moreover, by analyzing the "crossed cultures" of different mutants with various markers, heterokaryosis is reported although clamp connection is absent in this species.

Results from cytological studies showed that vegetative cells of *V. volvacea* were multinucleate and most of basidiospores were uninucleate but some contained more than one nucleus. Enucleate spores were also observed. Although the vast majority of mature basidia were tetrasporic, different spore patterns on basidia were found under the scanning electronic microscope. In addition, variation in nuclear DNA content was investigated by means of microphotometer. Based on the results from cytological studies, unequal distribution of meiotic nuclei and the formation of aneuploids could account for the phenotypic variation in *V. volvacea*.

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ABBREVIATIONS

PDA:	potato dextrose agar medium
CM:	complete medium
MM:	minimal medium
CY:	cycloheximide
CV:	crystal violet
MG:	malachite green
AC:	acriflavine
EB:	ethidium bromide
EDTA:	ethylene diamine tetraacetic acid
DAPI:	4',6-diamidino-2-phenylindole
MIC:	minimum inhibitory concentration
LC_{50}:	medium lethal concentration
cyR:	cycloheximide resistant mutant
cvR:	crystal violet resistant mutant
mgR:	malachite green resistant mutant
asp $^{-}$:	aspartate-requiring mutant
sp:	spontaneous mutant

Chapter 1: General introduction

Volvariella volvacea (Bull ex Fr.) Sing., commonly known as the straw mushroom, belongs to the family Pluteaceae of the Basidiomycetes (Singer, 1975). As reported by Chang (1977), *V. volvacea* was first cultivated in China. In the 18th century, someone in the Nanhua Temple (China) gradually or accidentally developed the primitive cultivation method of this mushroom on paddy straws. Therefore, *V. volvacea* has also been called the "paddy straw mushroom", "Straw mushroom" or "Chinese mushroom".

In recent decades, some edible mushrooms, macrofungi that produce fresh fruit bodies, have become more popular due to their excellent flavour and high nutritional values (Hatanaka *et al.*, 1974; Crisan and Sands, 1978; Cheng, 1979; Chang, 1980; Quimio, 1981; Li and Chang, 1982; Chang and Miles, 1989) and economic and biotechnological significance (Esser, 1977; Flegg, 1987; Elliott, 1988; Horgen and Anderson, 1989; Wood, 1989). On dry weight basis, mushrooms normally contain 19-35% protein as compared to 7.3% in rice; 13.2% in wheat; 39.1% in soybean and 25.2% in milk (Chang and Miles, 1989). In addition, the proteins of commonly cultivated mushrooms contain all nine amino acids essential in human nutrition.

Medicinal effects of edible mushroom which have attracted people's attention (Chang and Miles, 1989) include haematological effects (Lin *et al.*, 1973; Liener, 1979; Tsuda, 1979); antiviral activities (Kleinschmid, 1972; Suzuki *et al.*, 1973; Takehara *et al.*, 1979); antitumor actions (Misaki *et al.*, 1986; Mori and Takehara, 1989; Mori *et al.*, 1987;1989) and cardiovascular and renal effects (Lin *et al.*, 1973; Tam *et al.*, 1986). Volvatoxin A, a lectin isolated from *V. volvacea*, can reduce haemolytic activity toward Group O red blood cells (Lin *et al.*, 1973). In addition, polysaccharides, such as a cold alkali-extracted glucan (degree of branching 1:5) isolated from the fruiting body of *V. volvacea*, have a potent antitumor activity against Sarcoma 180 solid tumors implanted in mice (Misaki *et al.*, 1986).

As reported by Wood and Smith (1987), eight species of edible basidiomycete fungi are cultivated in significant quantities in various regions of the world. They are *Agaricus bisporous*, *Lentinus edodes*, *Volvariella volvacea*, *Flammulina velutipes*, *Pleurotus* spp., *Pholiota nameko*, *Auricularia* spp. and *Tremella* spp. Total global production is now in excess of two million tones fresh weight per year (Chang, 1987).

Global production value at producer prices is difficult to estimate exactly because individual species command different prices in different world regions, but total value is certainly in excess of US$ 1200 million per year. This can be

contrasted to global enzyme production value of around US$ 180 million per year, and for microbial pesticides of around US$ 3-6 million per year (Wood, 1989).

Based on the total production in the world, *Agaricus bisporus* is the most important with over one million tonnage output per year, mainly cultivated in Europe and the United States. *Volvariella volvacea* is the third most important edible fungus and is popularly cultivated in Southeast Asia (O'Brien, 1989; Chang and Tan, 1989). Estimated world production of *V. volvacea* in 1986 was 178 thousand metric tons in terms of fresh equivalent weight accounting for about 8.2% of world production of edible mushrooms (Chang, 1987).

With regards to the sexual patterns of edible mushroom, they can be broadly classified into either homothallic species or heterothallic species (Raper, 1978; Chang, 1982). In heterothallic species, cross mating between homokaryotic mycelia is required to complete the sexual cycle. The homothallic pattern of sexuality in Basidiomycetes may be one of two types, primary or secondary. For Primary homothallism, a self-fertile mycelium is developed directly from a single spore with a single meiotic nucleus. The presence of incompatibility factors in such mycelia has not been detected. However, in the secondary homothallism, the spore contains two compatible meiotic nuclei. The self-fertile mycelium is developed directly from a binuclear spore with two different mating types.

Primary homothallism is represented by the straw mushroom *V. volvacea*. Chang and associates were the first to report the sexual pattern of this species, which is able to complete its life cycle when mycelium is derived from a single meiotic spore, i.e. self-fertile without crossing (Chang and Chu, 1969; Chang and Ling, 1970; Chang, 1972). However, great variations among monosporous isolates were first reported by Chang and Yau in 1971, and confirmed later by Li and Chang (1979) and Chang *et al.*(1981). At present, the mechanisms of variation and sexual pattern of this mushroom remain uncertain. Thus, it is worthwhile to investigate them experimentally.

In order to study the pattern of sexuality and explain the possible sources of variation in *V. volvacea*, two strategical approaches, genetical and cytological studies were carried out. The first objective was to induce, select and identify a number of mutants which could be used in genetical studies. Ultraviolet (UV) irradiation, acriflavine and ethidium bromide were employed as the mutagenic agents to increase the mutation frequency. Cytological methods , such as light and electron microscopic examinations, autoradiography and microphotometry were conducted to study the nuclear behaviour in hyphae and basidiospores. Different spore patterns in the basidium were also investigated.

The data obtained in the present studies may give a better understanding of variation in monosporous isolates and the homothallic nature of this species. In

addition, the nuclear number and DNA content of basidiospore, hyphal cells and the spore pattern in basidium were elucidated. The possible sources of variation in *V. volvacea* can also be investigated. These experimental results could contribute to the development of some meaningful programmes for strain improvement in the straw mushroom.

Chapter two : Literature review

2.1. Introduction

V. volvacea has been cultivated for many years and has become the third most popular mushroom in the world. Literature available on this species includes its cultivation technology (Chang, 1964; 1965a; 1978b; Chang and Yau, 1970; Kohlii, 1987; Vela and Martinez-Carrera, 1989), morphology (Chang, 1965b; Chang and Yau, 1971; Li, 1982) and physiology (Chang and Yau, 1970; Kurtzman and Chang-Ho, 1982; Misaki *et al.*, 1986; Huang *et al.*, 1989). A full elucidation of sexuality and an understanding of variation depends upon the availability of genetical and cytological studies of *V. volvacea*. However, they are so far either fragmentary or have been interpreted from studies of a different species (Elliott and Challen, 1985).

2.2. Sexuality in higher fungi

Sexuality in the fungi has long been recognized as one of the most perplexing yet intriguing facets of the biology of microorganisms (Raper, 1966; Chang and Miles, 1989; Elliott, 1990). As early as 1904, Blakeslee discovered the obligatory cross-mating in the Mucorales. As a result of these studies, the fungi have been revealed as extremely versatile organisms in all aspects related to sexuality.

Sexual pattern is the nature of events leading to fertility. Its nature defines the potentialities and limitations for developing new strains through breeding. Now the sexual pattern in most of the cultivated edible fungi has been characterized (Raper, 1978; Elliott, 1979b, 1982).

Chang (1982) pointed out that although the process of sexuality is complicated by nutritional and physiological conditions, the genetic composition is the most critical factor determining both the occurrence and the morphology of the fruit bodies in edible mushrooms.

Sexual reproduction in higher fungi includes three important stages (Fig.2.1). The first essential stage is plasmogamy which is the fusion of cytoplasm of the two mating individuals. By plasmogamy the nuclei from two strains are brought together in a common cytoplasm. The second essential stage is known as karyogamy or nuclear fusion. The third essential stage is meiosis in which the chromosome number is reduced from the diploid to the haploid number. The product of meiosis is a tetrad. Through the process of sexual reproduction, genetic recombination and segregation may occur.

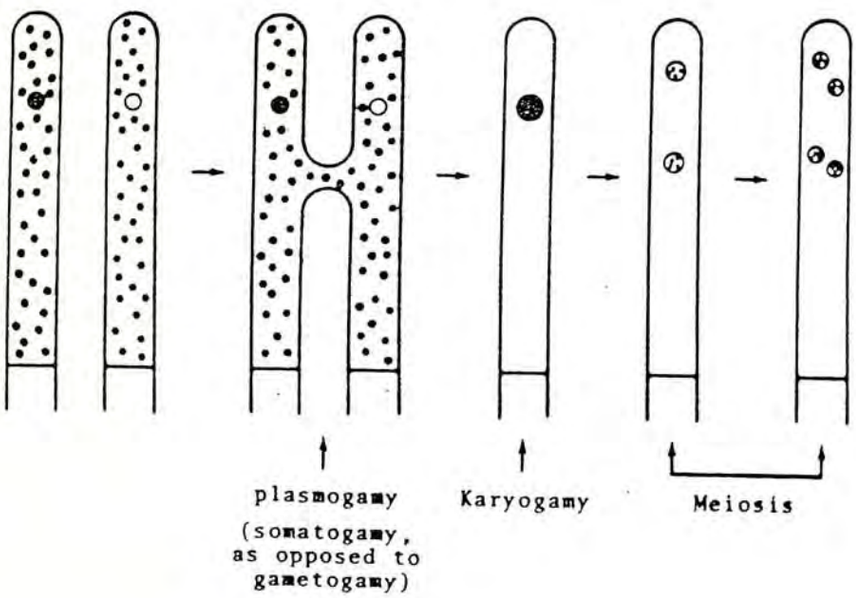


Fig. 2.1. Three stages of sexual reproduction in fungi (Chang, 1982)

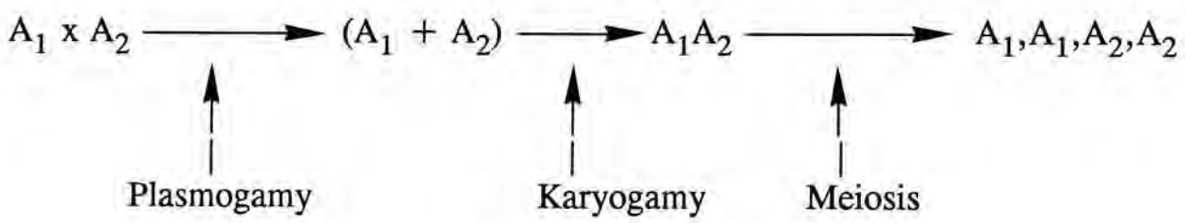
The higher fungi contain self-fertile, self-sterile and cross-fertile species. The common patterns of sexuality are heterothallism and homothallism. Generally, heterothallism predominates in the Basidiomycetes. Of the few hundred species in which the pattern of sexuality has been worked out, about 65% are heterothallic under bifactorial control and 25% are heterothallic under unifactorial control. The remaining 10% are either primary or secondary homothallic forms (Raper, 1966; Burnett, 1975).

2.2.1. Heterothallism

The term heterothallism was first used by Blakeslee in 1904 (cited from Raper, 1966) to describe the condition of sexual reproduction in certain species of the Mucorales. In heterothallic species, plasmogamy in cross-mating between different homokaryotic mycelia is necessary to establish the fertile dikaryotic mycelium. There are two mating systems :

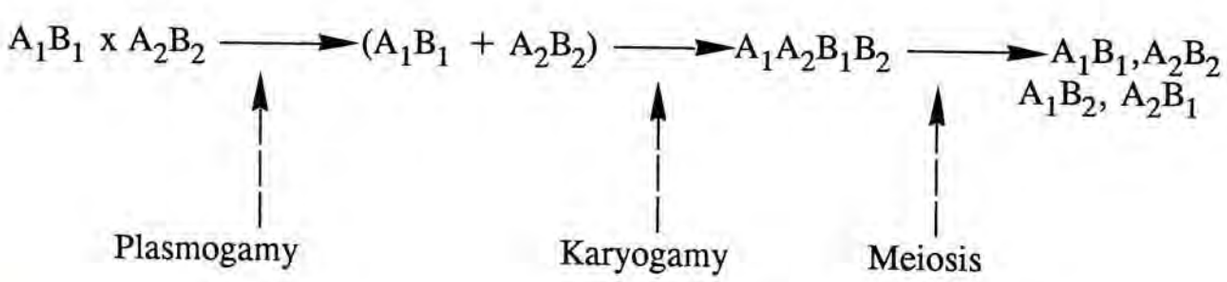
(1) Bipolar mating system in which the mating competence is determined by incompatibility factors of a single series, the A factor. The main processes of bipolar system may be summarized as follows (Chang, 1982):

Homokaryons	Dikaryon	Transient diploid	Homokaryotic progeny



(2) Tetrapolar mating system in which the mating competence is determined by incompatibility factors of two series, A and B, which assort and segregate independently at meiosis. The essential steps of this kind of mating system can be represented as follows (Chang, 1982):

Homokaryons	Dikaryon	Transient diploid	Homokaryotic progeny



Incompatibility is the restriction that prevents two genotypes from coexisting in the same cytoplasm (Birgittelane, 1981). Two different systems of incompatibility were described by Esser (1971): (1) Homogenic incompatibility: in this system karyogamy is prevented when nuclei carry identical incompatibility factor(s) so that inbreeding is prevented and outbreeding is encouraged; (2) Heterogenic incompatibility: the genetic prerequisite of heterogenic incompatibility is exactly opposite to that of the homogenic system: karyogamy is prevented when nuclei carry non-identical incompatibility factor(s), so that outbreeding is prevented and inbreeding is encouraged (Day, 1978; Birgittelane, 1981). The occurrence of these two incompatibility systems may be either independent or in combination to mediate the outcome of non-self hyphal fusions.

2.2.2. Homothallism

Two types of homothallism are found among self-fertile species: (1) primary homothallism in which a homokaryotic mycelium, established from a spore containing a single meiotic nucleus, has the potential to progress through heterokaryosis to complete the sexual cycle, and (2) secondary homothallism in which a fertile dikaryotic mycelium is established from a basidiospore carrying two meiotic nuclei of different mating types (Raper, 1966).

In brief, mating reactions in a heterothallic strain are determined by one or two sexual incompatibility factor(s) (Fincham and Day, 1971). However, a

homothallic fungus is one which is able to complete its life cycle when the mycelium is derived from a single meiotic spore, i.e. self-fertile (Oliver, 1953).

The sexual patterns in some basidiomycetes are summarized in Table 2.1.

Table 2.1. Sexual patterns in some basidiomycetes.

Species	Sexuality	
	Incompatibility factor(s)	Type
Agaricus bisporus	A	secondary homothallism
Agaricus bitorquis	A	bipolar heterothallism
Armillaria mellea	A, B	tetrapolar heterothallism
Auricularia auricula	A	bipolar heterothallism
Auricularia polytricha	A	bipolar heterothallism
Coprinus cinereus	A, B	tetrapolar heterothallism
Coprinus fimetarius	A, B	tetrapolar heterothallism
Coprinus sterquilinus	A, B	primary homothallism
Flammulina velutipes	A, B	tetrapolar heterothallism
Lentinus edodes	A, B	tetrapolar heterothallism
Pholiota nameko	A	bipolar heterothallism
Pleurotus ostreatus	A, B	tetrapolar heterothallism
Pleurotus sajor-caju	A,B	tetrapolar heterothallism
Schizophyllum commune	A, B	tetrapolar heterothallism
Volvariella bombycina	None*	primary homothallism
Volvariella volvacea	None*	primary homothallism

References: Raper (1966; 1978); Chang (1982); Duncan (1982); Lo and Chen (1989); Chiu and Chang (1987).

* not incompatibility factors have been detected yet.

2.2.3. Sexuality of *Volvariella volvacea*

The sexuality of *V. volvacea* has been considered as primary homothallism. Characterization of its sexuality is based on the self-fertility of most monosporous isolates. Moreover, meiosis in the basidium followed by the migration of one of the four meiotic nuclei to each of the four basidiospores is also an important cytological evidence (Chang and Chu, 1969; Chang and Ling, 1970; Chang and Yau, 1971; Chang, 1972). However, no incompatibility factor has been found at present.

Figure 2.2 shows the life cycle of *V. volvacea* (Chang and Yau, 1971). Basidiospores were uninucleate and haploid. After spore germination, a multinucleate hypha was formed. Chlamydospores, the vegetative spores, were born on specialized swollen cells of the multinucleate mycelium. After pinhead, tiny button, button, egg and elongation stages, a mature fruit body was produced.

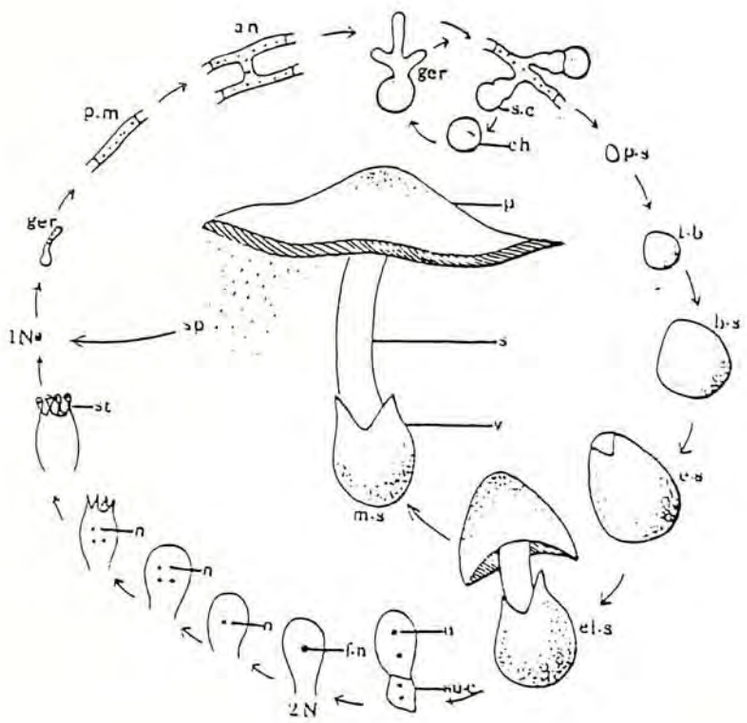


Fig. 2.2. Life cycle of *V. volvacea*.

an = anastomosis of monosporous hyphae; *b.s* = button stage; *ch* = chlamydospore; *e.s* = egg stage; *el.s* = elongation stage; *f.n* = fusion nucleus; *ger* = germ tube; *n* = nucleus; *1N* = haploid nucleus; *2N* = diploid nucleus; *p* = pileus; *p.m* = primary mycelium; *p.s* = pinhead stage; *s* = stipe; *s.c* = swollen cell; *sp* = spore; *st* = stigma; *su.c* = supporting cell; *t.b* = tiny button; *v* = volva (Chang and Yau, 1971).

Several hypotheses have been proposed to explain the self-fertile and self-sterile phenomenon that occurred in the progenies. Chang and Yau (1971) suggested that self-fertility in the majority of the progeny resulted from the presence of an incompatibility factor composed of two linked genes, A_1 and A_2, that operate together as two alleles of one factor. They further suggested that these mating factors, A_1 and A_2, were linked fairly strongly. Occasionally, unequal crossing-over may occur. A_1 and $A_1A_2A_2$ or A_2 and $A_1A_1A_2$ recombinants are produced. All of those recombinants are sterile. But A_1 or A_2 isolates are atypical and $A_1A_2A_2$ or $A_1A_1A_2$ isolates are normal in appearance. Up to date, however, such incompatibility factors have not been found in *V. volvacea*.

Because self-sterility was usually coincidental with aberrant mycelial morphology, an alternative explanation for the observed incidence of self-sterile, cross-fertile progeny in *V. volvacea* is a disruption by secondary genetic controls. The segregations of recessive mutant genes that alter morphology and block fruiting in a polygenic system have been found in other Basidiomycetes (Raper and Krongelb, 1958; Lemke, 1969; Perkins and Raper, 1970). However, it is difficult to explain the fact that some self-sterile progenies grow normally and have normal morphology.

In addition to these explanations, Elliott and Challen (1985) proposed a new secondarily homothallic model with a diploid / tetraploid nuclear cycle for *V. volvacea* and *V. bombycina*. However, no cytological evidence has been reported in this genus. Furthermore, Elliott and Challen (1985) subdivided the progeny of *V. bombycina* into two classes based on the distinct phenotypes including colonial morphology, growth rate and fertility. However, one of the twenty Type A mycelia tested in their case was exceptional (Chiu and Chang, 1987). On the other hand, using gamma irradiation as the tool of investigation, Quaye (1987) concluded that *V. volvacea* is a primary homothallic species and the basidiospores are haploid based on the radiation survival curve.

At present, the characteristics of the life cycle and the sexuality in *V. volvacea* are implied by some cytological evidence and analysis of the fertility pattern. It is well known that mutant markers are very important and useful in genetical analysis. However, experiments to analyze mutant markers in their progenies from basidiospores of fruiting bodies have not been conducted in this mushroom. At present, the mechanism of its sexuality is still not fully understood.

2.3 Induction and selection of genetic markers in higher fungi

Mutant genes may serve as markers which are important and useful in genetical analysis of any organism. The first mutants employed in a genetical experiment with a fungus were the spontaneous morphological mutant, *piloboloides* type V. normal sporangiophore and mating types, mt^+/ mt^- in *Phycomyces blakesleeanus*. Spontaneously occurring characteristics of this type plus colour mutants and pathogenicity reactions were virtually the only kinds employed until the advent of induced biochemical mutants (auxotrophs) in the classic experiments of Beadle and Tatum in 1941 (Burnett, 1975).

In some cultivated mushrooms, auxotrophic markers are of immediate use in a breeding programme as they can be exploited in the Petri dish (Raper et al., 1972). However, the induction and selection of auxotrophs are both difficult and time-consuming because of the multinucleate nature of hyphal cells and the binucleate spores of some mushroom (Pelham, 1967; Wang, 1972). Challen and Elliott (1987) pointed out that it was easier to identify resistant markers than auxotrophs as selective methods can be used. Actually, both auxotrophic and resistant markers are widely used in the present genetic studies.

Generally, mutants can be classified sa either spontaneous or induced. Spontaneous mutants can occur in all organisms but at an extremely low frequency. In the filamentous fungi, it is difficult to measure the spontaneous mutation rate, especially in those higher filamentous basidiomycetes that do not produce uninucleate asexual spores. Most of the studies of spontaneous mutation rate have been made with *Neurospora crassa*. Rough estimates of forward mutation rates vary somewhat with the allele. For *N. crassa*, a value of 1-10 per 100 million macroconidia was found in the literature (Chang and Miles, 1989). Recently, spontaneous mutation frequencies to carbendazin resistance were estimated at less than 1×10^{-9} in *Pseudocercosporella herpotrichoides* (Hocart *et al.*, 1990). These estimates are consistent with the finding of previous authors (Fehrmann *et al.*, 1982).

Since spontaneous mutations are rare, techniques of mutagenic treatments are used to increase the frequency of mutation, such as physical and chemical mutagenesis.

2.3.1. Physical mutagenesis

Mutation rates can be very greatly increased by certain kinds of irradiations. Among radiations, ultraviolet light is effective, and so is any kind of ionizing radiation such as x-rays, gamma rays and fast neutrons.

The ultraviolet (UV) lamp has been an extremely popular experimental tool in molecular and microbial genetics partly due to its convenience in use but also its relative safety. The maximum absorption of UV by DNA is at a wavelength of 254nm. Maximum mutagenicity also occurs at 254nm. The main mutagenic effect of UV is the induction of dimerization of pyrimidine. The consequence of dimer formation is an inhibition of normal DNA synthesis since the dimers are responsible for gaps in the strands of DNA that are synthesized. This method of mutagenesis is different from the mode of action of ionizing radiation such as x-rays, which causes alteration in the bases of the DNA and breaks in the DNA strands (Chang and Miles, 1989; Gardner and Snustad, 1984).

A series of reports have shown the effects of UV-irradiation on *Neurospora crassa* (Schroeder, 1970), *Saccharomyces cerevisiae, Schizosaccharomyces pombe*, (Martin *et al.*, 1981; Lawrence, 1982), *Aspergillus flavus* (Osman *et al.*, 1989). , *Penicillum notatum, Ustilago maydis* and *Schizophyllum commune* (King, 1974; Shneyour *et al.*, 1978; Fincham and Day, 1971). The effects of ultraviolet radiation on *Candida albicans* have also been investigated. It was found that inactivation of non-budding cells by ultraviolet irradiation is determined in part by (i) their post-irradiation incubation temperature, (ii) opportunity for exposure to long chain fatty acids or sterols

and (iii) the nutritional quality of their post-irradiation growth medium. These cultural conditions do not affect the viability of cells which are budding when irradiated or the frequency of mutants among the survivors of irradiated, unbudded populations. Resistance to the membrane-specific fungicidal antibiotic, amphotericin B, changes the composite pattern of survival responses of UV treated unbudded cells to the three post-irradiation conditions. Acquisition of amphotericin B resistance does not alter the mutability of such cells nor the vulnerability of budding cells to UV irradiation. Differences in susceptibilities of cells to polyene antibiotics are known to be determined by differences in the composition and structural arrangement of their membrane (Sarachek and Pettriess, 1974).

Post-irradiation growth on sterols or long chain fatty acids promotes recovery of *C. albicans* from UV-induced lethal damage. It was suggested that lipids remediate an UV-induced derangement of the structure of the cell membrane critical for the initiation of cell division (Sarachek and Higgins, 1972). Moreover, effects of caffeine on genetic response of *C. albicans* to UV-irradiation were studied (Sarachek *et al.*, 1970; Sarachek and Bish, 1976). Inhibition of UV mutagenesis at high UV doses at 37°C indicated that recombination repair in *C. albicans* is sensitive to caffeine. On the other hand, comparison of cellular inactivation and mitotic recombination induced by ultraviolet radiation between aneuploid and euploid strains of *C. albicans* was also carried out by Rhoads and Sarachek in 1984.

It was reported by Grindle (1984) that mutants of *Neurospora crassa* were induced with ultraviolet light and isolated on synthetic medium containing the dicarboximide fungicide vinclozolin. *Vin* (vinclozolin-resistant) mutants were more resistant than wild type to the fungicides, chloroneb, dicloran, iprodione, procymidone, quintozene and vinclozolin. Resistance to dicarboximide fungicides was associated with reductions in radial growth and conidiation on synthetic media, especially on medium supplemented with sodium chloride.

Fungicide resistant mutants of *Agaricus bisporus*, a cultivated mushroom, have the potential as markers for use in the production of novel hybrid mushroom strains and disease control programmes. Challen and Elliott (1987) used UV mutagenesis to induce resistance to four fungicides (benodanil, carboxin, imazalil and tridemorph) in different strains of *A. bisporus*. All mutants showed enhanced *in vitro* tolerance to specific fungicides when compared with the parental strains. Cross resistance was found to occur between mutants with specific resistance to either benodanil or carboxin. The tridemorph mutants were altered in colony morphology and were not fertile. All other mutants produced were fertile and they retained the agronomic characteristics of the strains from which they were derived. Furthermore, mutants resistant to carboxin could fruit and yield well at concentrations of the fungicide that substantially reduced the yield of the sensitive parent strains. These trials suggested that resistant spawns coupled with fungicide applications can be used in the control of fungal diseases (Challen *et al.*, 1989a; 1989b).

Selection and genetic analysis of antibiotic-resistant mutant strains induced by UV-irradiation in *Agrocybe aegerita* have been reported by Labarere *et al.*, (1989). Four antibiotics used were chloramphenicol and three aminoglycosides (neomycin, paromomycin and tobramycin). Studies on cross-resistances showed that neomycin-resistant strains were also resistant to paromomycin. Moreover, genetic analysis of antibiotic-resistance was performed for several mutant strains by crosses with wild-type sensitive strains. Segregation analysis of the antibiotic-resistant characteristic showed that resistance had a single gene mutation determinism in most cases. Dominance tests demonstrated that mutations were recessive. These recessive single mutations could be used for mapping the genome of *A. aegerita*, or for establishing conditions for protoplast fusion.

It is known that the spores of *Pleurotus* species are responsible for adverse allergic reactions and respiratory disorders in some workers of the *Pleurotus* growing enterprises. They can affect the external aspect of the

basidiocarps after storage by deposition of a white powder (the spore) and germination (Leallara, 1977). Sporeless mutants and poorly-spored mutants obtained by ultraviolet mutagenesis in the two most cultivated French species: *Pleurotus ostreatus* and *Pleurotus pulmonarius* were studied by Imbernon and Labarere (1989). They found that the morphology of the mutant strains was always atypical (central stipe too big compared with cap size, cup-shaped or cone-shaped caps). In small-scale cultures the yields of the mutant strains were lower than their parental strains.

Quaye (1987) studied the effects of gamma irradiation on the basidiospores of *V. volvacea*. By analyzing the radiation survival curve, he concluded that basidiospores are haploid. Barroso *et al.* (1988) investigated the effects of UV irradiation on germination of *V. volvacea* basidiospores. The analysis of the survival curve of irradiated basidiospores was according to the haploidy of *V. volvacea* basidiospores. Chloramphenicol and tetracycline were found to be two inhibitors of spore germination and hyphal growth in *V. volvacea*. UV irradiation increased the frequency of chloramphenicol and tetracycline resistant strains obtained after germination of the irradiated basidiospores. Analysis of the stability of the resistant phenotypes showed a loss of the resistance after subculturing. Accordingly, the resistance may be extrachromosomal mutations and physiological adaptation instead of nuclear mutation.

2.3.2. Chemical mutagenesis

There are many chemical substances which may act as mutagens. Fincham and Day (1971) presented a clear account on chemical mutagens in particular reference to the fungi.

Generally, chemical mutagens can be divided into two classes: (1) those that are mutagenic to both replicating and nonreplicating DNA, such as the alkylating agents, and (2) those that are mutagenic only to replicating DNA. The

latter class includes (a) the acridine dyes which bind to DNA and increase the probability of making mistakes during replication, and (b) base analogs, purines and pyrimidines, with structures similar to the normal bases of DNA. The base analogs must be incorporated into DNA chains in the place of normal bases during replication to exert their mutagenic effects. Gupta *et.al.* (1981) isolated nineteen fungi from different soil samples on the basis of clear zones formed on Rose Bengal Cellulose agar medium. In shake flasks, the isolate K_1, which was identified as a *Phoma* species, gave 12.1 units/ml of CMCase activity. After N-methyl-N'-nitro-N-nitrosoguanidine treatment of isolate K_1, a mutant KM_2 was obtained. This mutant differed morphologically from the parental strain on RBCA medium and gave 36.2 units/ml of CMCase activity. On the other hand, the yield of ß-glucosidase was increased about 8-fold in the mutant KM_7 and was about 68% higher than the level found in the standard organism *Trichoderma viride* QM 9414.

Extranuclear chloramphenicol resistance mutations in the Basidiomycete *Sistatrema brinkmannii* were reported by Anderson and Cenedese (1984). In their experiment, four chloramphenicol resistance mutations were induced in homokaryons of *Sistotrema brinkmannii* with N-methyl-N'-nitro-N-nitrosoguanidine. Then they used a heterokaryon test to determine whether the mutations were nuclear or extranuclear. Dikaryons were recovered from sexually compatible pairings of chloramphenicol-resistant and sensitive strains which also carried chromosomal markers (mating-type and auxotrophic). The homokaryotic components of the dikaryons were recovered from vegetative cells by the production and regeneration of protoplasts. The homokaryons derived from protoplasts were tested for chloramphenicol resistance / sensitivity as well as for the chromosomal markers. In heterokaryon tests, the chloramphenicol resistance / sensitivity determinants re-associated with the chromosomal markers. Only a single recombinant with respect to chromosomal markers was observed among a total of 220 homokaryons analyzed. All pairs of the same chromosomal markers produced numerous recombinants in sexual crosses. These results indicated that the chloramphenicol resistance mutations were

extranuclear.

By using N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis, Imbernon and Labarere (1989) successfully induced sporeless mutants and poorly-spored mutants in *Pleurotus*. However, no morphological or auxotrophic mutants could be obtained from protoplasts in *Volvariella volvacea* by using this chemical mutagen (Mukherjee and Sengupta, 1986).

Nitrous acid (NA) is a potent mutagen that acts directly on DNA to produce a change in one of the bases. It does this by reacting with primary amino groups to give hydroxyl groups. Ethyl methanesulfonate (EMS) alkylates both adenine and guanine and causes some depurination of DNA. Sarachek and Bish (1976) have reported that at comparable levels of survival, NA is more effective mutagen than EMS in *Candida albicans*. However, they also showed that UV induced a much greater frequency of mutants than EMS and NA in this species.

2.3.3. Biological mutagenesis

Biological mutagenesis generally refers to the induction of mutants with biological method instead of by physical or chemical mutagenesis.

Transfer of a heritable character by a naked DNA is called transformation (Mishra, 1985). Since Avery and co-workers in 1944 first elucidated its mechanism, this genetic process has captured the imagination of biologists because of the immense possibilities it promises in directing the changes in the genetic makeup of organisms.

The transfer of recombinant DNA molecules into fungi has been achieved in a growing number of filamentous fungi and in yeast (Mishra, 1985; Hynes, 1986). As pointed out by Horgen and Anderson (1989), three approaches have been used. The first approach is the transformation of auxotrophs to

prototroph by the introduction of a complementary gene. For example, the *ura-5* gene of *Podospora anserina* (Begueret *et al.*, 1984) was first obtained by complementation of *E. coli* mutations and then subsequently used in fungal transformation. Similarly the *trp C*, *trp-1* and *arg B* genes of *Aspergillus nidulans* and *Neurospora crassa* (Berse *et al.*, 1983; Hynes, 1986; Schectman and Yanofsky, 1983) were also obtained by this way.

The second approach is the introduction of a gene for antibiotic resistance from a prokaryotic source. It was reported that a chimeric plasmid containing the *E. coli* R factor-derived chloramphenicol resistance (cam^r) gene has been constructed, which conferred resistance to a very high level of chloramphenicol (500 μg/ml) on yeast cells (Mishra, 1985). The yeast cells carrying the chimeric plasmid produce an active chloramphenicol acetyl transferase enzyme, which is responsible for the antibiotic resistance. Other possible genes for similar use include benlate, cycloheximide and hygromycin B resistance (Hynes, 1986; Mooibroek, *et al.*, 1990).

The third approach is the addition of a new gene that alters the nutritional requirements of the fungus (Turgeon *et al.*, 1985). Within the gilled basidiomycetes, transformation has been achieved in *Schizophyllum commune* (Munoz-Rivas *et al.*, 1986).

The basic method for fungal transformation involves the formation of protoplasts generated by hydrolysis of the fungal wall with cell wall degrading enzymes (Hamlyn *et al.*, 1981; Peberdy, 1979; 1985; 1989). This is followed by the treatment with DNA in the presence of calcium and polyethylene glycol (Mishra, 1985; Hynes, 1986). On the other hand, successful transformation of lithium acetate-treated *N. crassa* (Dhawale *et al.*, 1984) is an important development worth pursuing in other species.

Horgen and Anderson (1989) have attempted to transform resistance to either neomycin or the aminoglycoside antibiotic G418 into *Agaricus*

brunnescens. They observed putative, but abortive, transformed *Agaricus* mycelium on selective plates. Moreover, they have also been working on a methodology of electric field pulsation for the uptake of their constructed vectors into *A. brunnescens* germinating basidiospores. This approach has been successful in injecting DNA into walled yeast cells and tobacco culture cells (Morikawa *et al.*, 1986).

Elliott (1990) and his coworkers are trying to isolate a gene for resistance to the fungicide, carboxin, to use in a transformation system. This work is also being done in the ink-cap species *Coprinus bilanatus*. It has already been demonstrated that a gene can be transferred from one *Coprinus* species to another.

It is worth emphasizing that protoplasts are important in both classical and molecular genetics. Most of the laboratories currently engaged in fungal genetics are using gene manipulation procedures based on protoplasts. Moreover, protoplast fusion is a technique whereby the genomes of different strains, either isogenic, non-isogenic, or of different species can be brought together as the first step towards recombination (Layton and Kuhn, 1988). Transformation of protoplasts provides opportunities for the manipulation of individual genes or specific DNA sequences derived from a given gene opening up the whole field of molecular genetics in fungi (Ferenczy, 1981; Ohmasa, 1986; Ogawa *et al.*, 1988; Peberdy, 1978; 1989).

In spite of the economic importance of *V. volvacea*, little effort has been made to genetically improve the strains. Up to date, no mutants have been obtained by biological mutagenesis although there are limited reports on producing protoplasts from the mycelium of this edible mushroom (Santiago, 1982; Chang *et al.*, 1985).

2.4. Nuclear behaviour in edible fungi

Nuclear behaviour furnishes an important clue as to the genetic problems and life cycles of the fungi (Chang, 1978a). The general feature of the cells of edible fungi is similar to that of other fungi.

2.4.1. Somatic division

Division of somatic nuclei in vegetative hyphal cells is not well understood. It has become a subject of dispute in the mycological literature. Lu (1962), Ward and Ciurysek (1961, 1962), Shatla and Sinclair (1966) substantiated that somatic nuclei undergo classical mitosis with the aid of a spindle apparatus in fungal hyphae. However, Robinow (1957), Bakerspigel (1959), Saksena (1961) provided evidence in supporting the direct, amitotic division of vegetative nuclei.

It is now generally accepted that fungi are apparently heterogeneous for mechanisms of mitosis (Kowalski, 1966; Peng and Wu, 1972; Day, 1972; Lemke *et al.*, 1975; Poon and Day, 1976; Kühner, 1977; Heath, 1978; Heath, 1980a,b). Day (1972) proposed the models for mitosis in higher fungi. One of his models is called the "two-track" type by which chromosomes are aligned end-to-end in an elongated circle during metaphase and are then split and migrate to opposite pores during anaphase. On the other hand, nuclear migration and intracellular nuclear movement are also found in some fungi.

In the study of *V. volvacea*, figures of the two modes of somatic nuclear division were found in the same preparation and in several cases even within the same cell. Chang and Ling (1970) observed amitosis of constricted nuclei in *V. volvacea*. The spherical nucleus first increases in size and elongates (the dimensions of elongated nuclei range from 0.84 x 1.5 μm to 1.68 x 2.69 μm). Then a constriction starts to occur at the middle. The two apparently equal halves are pulled apart, connected for a moment only by a delicate strand of

chromatin material. As the daughter nuclei move apart, a beaklike part is drawn out at the point of disjunction. Each new nucleus soon resumes the spherical outline of an interphase constricted nucleus. This similar sequence of events has also been observed under the electron microscope in *V. volvacea* (Chang and Ling-Wong, 1974), in *Lentinus edodes* (Nakai and Ushiyama,1974) and in yeast nuclei (Moore, 1965).

Up till now, there have been no substantiated studies on the cytogenetics of *V. volvacea*. The problem of how a uninucleate, haploid spore in a homothallic species gives rise to a multinucleate mycelium is rather controversial. The interpretation of sexual and genetic behaviour in *V. volvacea* is much more difficult than in the heterothallic species.

2.4.2. Meiotic events

The most detailed and accurate accounts of meiosis in the fungi have come from studies of *Neurospora crassa*. McClintock (1945) and Singleton (1953) first described and documented the meiotic sequence in *N. crassa* with particular emphasis on the chromosome cycle. Cytological observations were made with iron-haematoxylin stained techniques. Raju's (1980) observation agreed generally with those of McClintock and of Singleton.

Although the meiotic events have been described in many species of fungi (Setliff *et al.*, 1974; Murakami and Takemaru, 1985; Hennon and Hansen, 1987), the time sequence of the events was first reported by Raju and Lu (1970) in *Coprinus lagopus*. It takes about 16 hours from the beginning of karyogamy to the completion of meiosis. Raju and Lu (1973) reported that meiotic divisions in *Coprinus* were in close synchrony. However, asynchronous meiotic divisions were found in some fungi, e.g. *Tremella rubromaculata* (Furtado, 1968), *V. volvacea* (Chang and Chu, 1969), *A. bisporus* and *S. rugoso-annulata* (Thielke, 1972).

There are several reports of basidiomycete species having two spores instead of four (Kemp, 1974) and genetic studies have been carried out on these species (Langton and Elliott, 1980). Ross and Margalith (1987) studied the nuclear behaviour in the secondary homothallic *Coprinus bilanatus*. This two-spored species was found to be similar in many respects to *A. bisporus* and *C. cinereus*. The major point of variation was in the timing of the movement of the four meiotic products into the developing basidiospores. In *C. bilanatus*, the spores are full sized and heavily pigmented before nuclei move into them. Two nuclei enter each spore. The apical to basal movements of meiotic nuclei a reported suggest that the alignment of the spindles at meiosis II may not be significant factor in the entry of nuclei into specific spores.

Atypical meiosis in the secondary homothallic fungus *Agaricus brunnescens* has been investigated by Spear *et al.*, (1983). Normal assortment in *A. brunnescens* usually results in complementary meiotic nuclei packaged in each spore. Thus, the majority of single-spore-derived isolates is heterokaryotic. However, homokaryotic multinucleate spores were found in their study. It might be due to the occurrence of non-disjunction during metaphase II. This would place sister chromatids in the same spore. Moreover, it might be expected to create some uninucleate spores. Since two uninucleate spores were observed in their study, the possibility of non-disjunction cannot be eliminated.

As pointed out by TeBeest *et al.*, (1989) the number of nuclei in spores is an important consideration when protoplast fusion or plasmid-mediated recombination experiments are conducted since it is necessary to isolate uninucleate cells. In their experiment the number of nuclei in spores of three species of *Colletotrichum* was observed by using the fluorescent dye acriflavine (Raju, 1986) or DAPI (4',6-diamidino-2-phenylindole). Although the results obtained from staining spores with these two dyes were nearly identical, it was found that DAPI was more convenient to use, more rapid and produced sharper images of nuclei, with less background, than acriflavine. Now fluorochromes have become more widely used in the study of fungal cytology (Butt *et al.*, 1989;

Lo and Chen, 1990). On the other hand, nuclear number in each cell of the hyphal system of fertile cultures of *Agaricus bisporus* were measured with the aid of Giemsa stain systematically (Wang and Wu, 1974). They proposed that the fruiting of the mushroom required a certain range of nuclear ratio.

In the study of basidia of *Collybia maculata var. schorzonerea*, Huffman (1968) found the increase in length of the basidium and nuclear volume follow very closely the periods of active synthesis of nuclear material during meiosis. The greatest increase in basidium length and total nuclear volume per cell occurs from the time of binucleate prefusion nuclei to the completion of the first division of meiosis. However, it differs from those in *V. volvacea* (Chang and Ling, 1970) where basidia at various meiotic stages happen to be quite regular in width, size, and shape. Only after the basidia become mature , the distal portion lengthens and the free ends flatten to give rise to four sterigmata. After nuclear fusion, with the enlargement of the basidium, the diploid nucleus also enlarges rapidly. It sometimes became almost twice the volume of the haploid nucleus (Chang and Ling, 1970). The young fusion nucleus of *V. volvacea* remains compact and does not enter into meiosis immediately. In other words, there is a brief resting period between the fusion of nuclei and subsequent divisions (Chang and Chu, 1969).

Li (1977) has reported that the haploid number of *V. volvacea* was nine, which differed from Juliano's result of four. Recently, some Chinese researchers found that the haploid number was eleven (Li *et al.*, 1991). Possible explanations for the differences are (1) different strains of the fungus having different chromosome numbers; or (2) the differences are due to inadequacy of techniques. Actually, Li's result (Li, 1977) is exactly the same as that reported by Chang and Chu (1969). During meiosis, chromosomal pairing is complete and no multivalent associations have been observed. Li (1977) also suggested that nuclear selection must have occurred. Only haploid nuclei remain, while all other nuclei (of various ploidies) are gradually eliminated during the selection process. All nuclei in the fruit body tissues are similar in shape and size. Li

further suggested that these nuclei were regarded as homogeneous-heterokaryotic, with one set of chromosomes but a different genetic constitution. However, this hypothesis remained to be further studied.

2.4.3. Electrophoretic karyotype

Recently, new techniques of preparing chromosomes from fungal protoplasts for pulsed field gel electrophoresis have been developed. The successful application of these techniques at resolving yeast chromosomes has paved the way for karyotype studies of other microorganisms including filamentous fungi (Miao and VanEteen 1990). It was reported by Brody and Carbon (1989) that an electrophoretic karyotype of *Aspergillus nidulans* was obtained using contour-clamped homogeneous electric field gel electrophoresis. Six chromosomal bands were separated, with two of the bands migrating as doublets. Using chromosomal DNA of *Schizosaccharomuces pombe* and *Sacccharomyces cerevisiae* as size standards, they estimated the sizes of the chromosomes to be between 2.9 and 5.0 megabase pairs (Mb) with a total genome size of approximately 31 Mb. Electrophoretic karyotype of *Neurospora crassa* (Orbach *et al.*, 1988), *Candida albicans* (Magee *et al.*, 1988) and *Phytophthora megasperma* (Howlett, 1989) were also determined.

Pulsed field gel electrophoresis has been applied to a number of *Pleurotus* spp. and strains to investigate chromosomal variation in this group of commercially important fungi. Up to 7 chromosomes ranging from 5.7 Mb to 2.2 Mb were reported to be separated by Fox and Peberdy (1990).

Chapter 3. Variations in monosporous isolates of *Volvariella volvacea*

3.1. Introduction

As mentioned in Chapter 2, *V. volvacea* has been considered as a primary homothallic species. Theoretically, a self-fertile mycelium derived from an uninucleate basidiospore should be homokaryotic (Raper, 1978). However, great variations among monosporous isolates of *V. volvacea* were first found by Chang and Yau (1971) and confirmed by Li and Chang (1979). It leads to different explanations on the sexuality pattern and to postulation of possible sources for variation in this edible mushroom.

The work reported here was undertaken to provide information about the variations in monosporous isolates of *V. volvacea*. The characteristics of monosporous isolates from five geographical strains were analyzed by examining the colonial morphology, linear growth rate of vegetative mycelia and fertility. A comparative study of extracellular cellulase activity between fertile and sterile monosporous isolates was also carried out. Because the fertility tests of the monosporous isolates so far reported were conducted in mushroom houses where it is possible for spores to be present in the air, which might be affect the results, the explanation of the results can be questioned. For precaution, a

special device was set up to conduct the fructification of *V. volvacea*.

3.2. Materials and methods

3.2.1. Strains and culture media

Five strains of *V. volvacea* were used in this experiment. Their source is listed in Table 3.1.

Table 3.1. Sources of *Volvariella volvacea* strains studied.

Strain	Sources
V_5	Tissue culture obtained from Thailand.
V_7	Tissue culture from field collection in Pelating Java, Malaysia.
V_{14}	Isolated from Tennamaran Estate, Batang Berjuntai Selangor, West Malaysia.
V_{35}	Hong Kong (=Ta Lung No.4).
V_{42-18}	Single spore isolate derived from Strain V_{42} (Hong Kong).

Two media were used: the complete medium (CM) (Raper and Miles, 1958) and synthetic potato dextrose agar medium (PDA). The complete medium consists of : dextrose, 20g/l; yeast extract, 2g/l; bacto-peptone (Difco), 2g/l;

KH_2PO_4, 0.46g/l; K_2HPO_4, 1g/l; $MgSO_4.7H_2O$, 0.5g/l; bacto-agar (Difco), 15g/l.

3.2.2. Monosporous isolation

Basidiospores were collected and isolated as described by Chang *et al.* (1981). Fresh spores were first collected on complete agar medium plates for about 10-15 seconds per plate. After incubation at 32°C for 48-72 hours, the germlings were isolated with a mechanical spore cutter (Raper, 1963) under the microscope. Each cut agar block with only one spore germling was transferred by means of a chisel pointed needle onto a complete agar medium.

3.2.3. Linear and radial growth rate test

The linear growth rates of the cultures of monosporous isolates were determined by measuring the progression of mycelial frontiers within horizontal glass tubes filled with 20 ml of complete agar medium. Five-day old mycelia were used for the test. All inocula were made uniform by means of 5 mm standard cork borer and all test tubes were incubated at 32°C. The linear growth rate of each isolate was tested in triplicate. Forty-eight hours after inoculation, initial lines were made and for another 5 days, the end points were determined.

For the radial growth rate determination, a 2mm mycelial agar block was

put at the centre of the plate (diameter 8.5cm) containing complete agar medium or potato dextrose agar medium. Radial growth was measured by day(s) after initial lines were made which were marked 24 hours after inoculation.

3.2.4. Fertility test

Fertility tests were conducted in cotton waste compost. Cotton waste is the byproduct of the textile industry. Its usage in mushroom cultivation involves the recycling of an industrial waste (Chang, 1978b).

The compost was prepared by mixing cotton waste mixed with 3% (w/w) calcium carbonate (industrial grade) and a suitable amount of water to give a moisture content of about 63% (Chang *et al.*, 1981). After thoroughly mixing, approximately 400g of substrate was filled into a plastic bag (15x25 cm) and sterilized by autoclaving at 1.25 kg/cm^2 pressure and 120°C for 30-40 minutes.

Six inocula from a 5-day old mycelial culture were cut with a 1 cm cork borer. Then they were inoculated into each sterilized compost bags and incubated at 32°C for 12 days. After 12 days, by which time there was complete colonization of mycelium on the substrate, the compost bags were opened to stimulate fruit body formation. The temperature was then maintained at 29°C and relative humidity at 90%. Ventilation and light were required for healthy fruit body development.

A monosporous isolate, which produced fruit bodies on all triplicate testing compost bags, was considered self-fertile. If there were still no primordia (pin heads) produced 25 days after opening the compost bag, it was scored self-sterile.

Some of the assigned self-sterile isolates were "crossed" to see whether they would be stimulated to become "fertile" again. The method used was as described by Chang and Yau (1971). Because there is no clamp connection between heterokaryotic hyphae in *V. volvacea*, the term "fertile" used here simply referred to the ability of paired mycelia to form sporocarp.

In order to prove that each single spore isolate can produce a mature fruit body, a special device was set up (refer to Fig. 3.1).

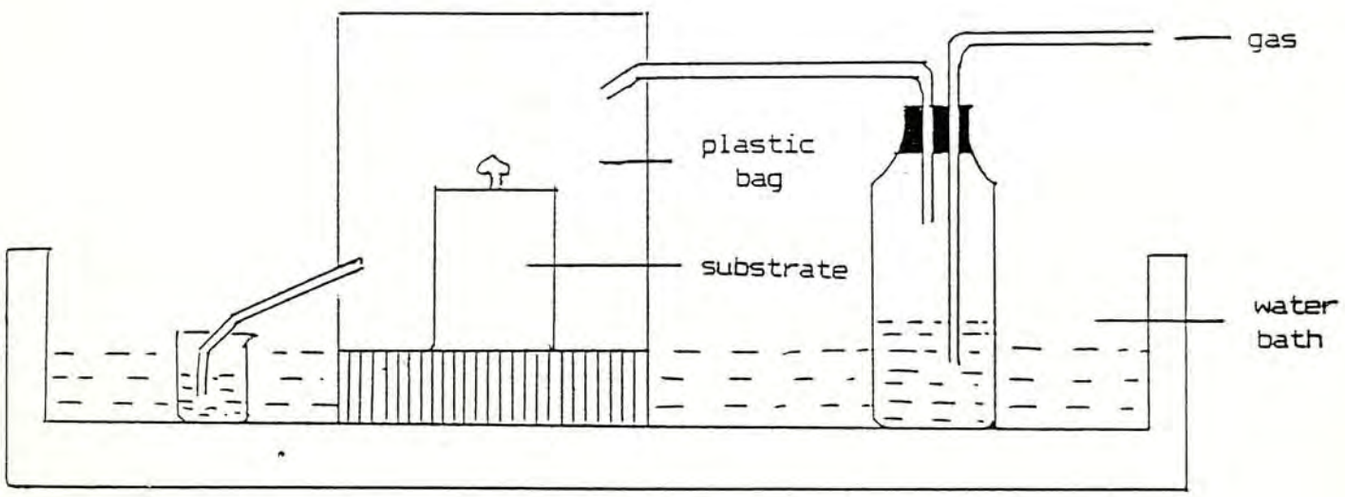


Fig. 3.1. Set up of a special device for fruiting of *V. volvacea*.

In this experiment, a compost bag with fully growing mycelium was put inside a transparent plastic bag. Before entering the plastic bag, the gas has to pass through the gas-washing bottle and all the spores in the air will deposit in the water. A hole was punched in the lower part of the plastic bag. This hole leads to a tube which can let the excess air pass out and facilitate the ventilation. This device can prevent the contamination by spores of other *V. volvacea* strains.

3.2.5. Determination of extracellular cellulase activity

Wild type strain $V_{42\text{-}18}$ and its six monosporous isolates were used to determine the extracellular cellulase activity. Their main characters are shown in Table 3.2.

Table 3.2. Monosporous isolates for enzyme assay.

Monosporous isolates	Remarks
V_{42-18}	Parental strain
F-1 F-2	Fertile isolate
SN-1 SN-2	Sterile isolate with normal colony morphology
SA-1 SA-2	Sterile isolate with abnormal colony morphology

The liquid medium used for enzyme assay was modified from the mushroom complete medium (Yoo *et al.*, 1988), in which carboxymethyl cellulose (CMC) is the carbon source substituted for the glucose. It had the following composition (per litre): carboxymethyl cellulose, 10g; yeast-extract, 2g; $MgSO_4.7H_2O$, 0.5g; KH_2PO_4, 0.46g; K_2HPO_4, 1g; asparagine 2g; thiamine, 120 μg; pH was about 6.8.

Two mycelial agar blocks cut by a 2mm cork borer were inoculated in 250 ml Erlenmeyer flask containing 50 ml sterilized medium.

The activity of extracellular cellulase was determined by the amount of soluble reducing sugar formed when the crude extract was incubated with carboxymethyl cellulose as substrate (Trigiano and Fergus, 1979). The mycelium-free filtrate was tested at 5 day intervals.

In the experiment, 0.5 ml of mycelium-free filtrate was transferred to a test tube containing 0.5 ml of 0.55% CMC citrate buffer solution (pH 5.0-5.2). After incubation at 40°C for 1 hour, 1 ml of the mixture and 2 ml of dinitrosalicylic acid (DNS) reagent (Miller, 1959) were added to the mixed solution and boiled for 15 min. On the other hand, filtrates reacting with only dinitrosalicylic acid reagent but not CMC-citrate-buffer solution were used as control. The optical densities of the solutions were determined with a spectrophotometer at the absorbance of 575nm, and reducing sugars assessed by comparison to a standard curve constructed on solutions for glucose of known concentration covering the range up to 300 μg / ml, giving a slope of about $2x10^3$. In addition, mycelia produced were harvested and dried (at 50°C for 72 hr) and the dry weight determined.

3.3. Results

3.3.1. Variations in colonial morphology

Colonial morphologies of monosporous isolates were observed and compared. They were divided into the "normal" and "abnormal" isolates (Fig.3.2.). Totally, 328 isolates were examined. It was found that variation of colonial morphology occurred in monosporous isolates although they were obtained from the same fruit body.

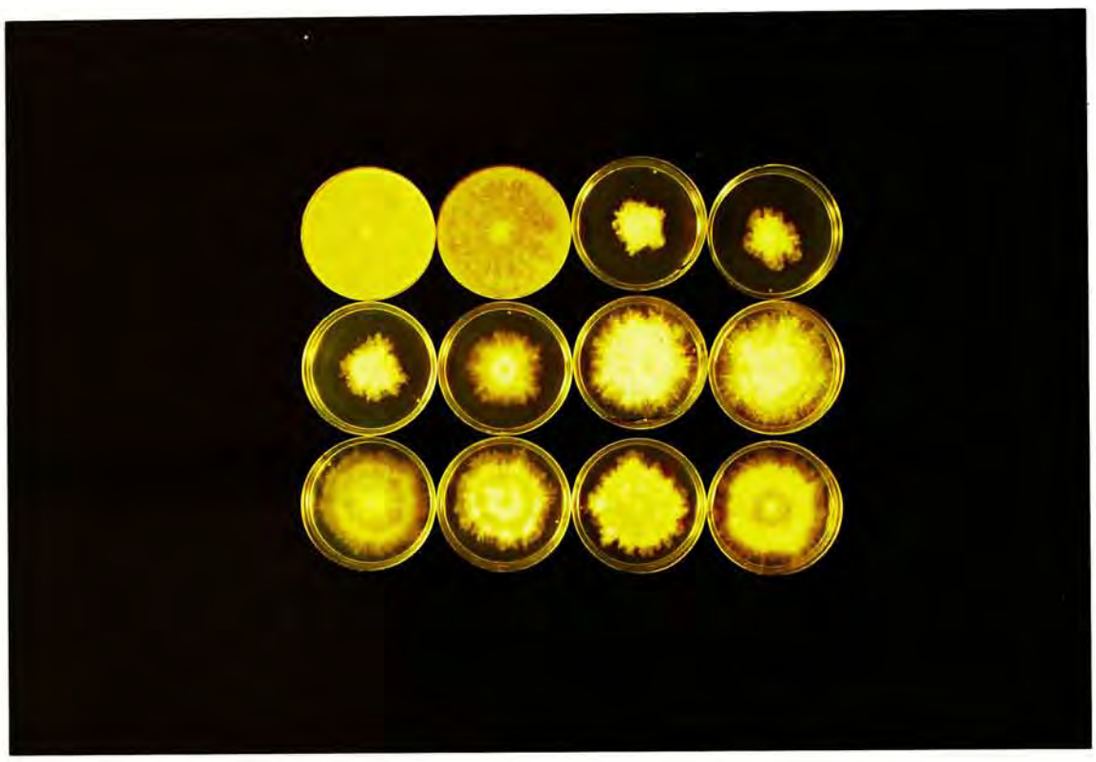


Fig. 3.2. Colonial morphology of some monosporous isolates of *V. volvacea*. The two plates at the left in the top row show the normal colonial morphology. The mycelia grow vigorously with abundant aerial and horizontal hyphae. All the other plates show the abnormal colonial morphology. The mycelia grow slowly with very few or even no aerial hyphae.

Table 3.3. Percentage of "normal" and "abnormal" colonial morphologies among monosporous isolates of *V. volvacea*.

Strain	No. of isolates observed	Colonial morphology "normal" No.	%	"abnormal" No.	%
V_5	42	35	83.33	7	16.67
V_7	38	34	89.47	4	10.53
	42	35	83.33	7	16.67
V_{14}	60	40	66.67	20	33.33
V_{35}	55	38	69.09	17	30.91
V_{42-18}	50	39	78.00	11	22.00
	41	31	75.61	10	24.39

(a) "Normal" colonial morphology: colonies with vigorously growing mycelia and abundant aerial and horizontal hyphae.

(b) "abnormal" colonial morphology: colonies with very few or even no aerial hyphae, or with compact mycelial forms.

3.3.2. Variations in linear growth rate.

Monosporous isolates from five strains showed variations in the linear growth. Some isolates grew faster or slower than their parental strain. (Fig.3.3.a and b).

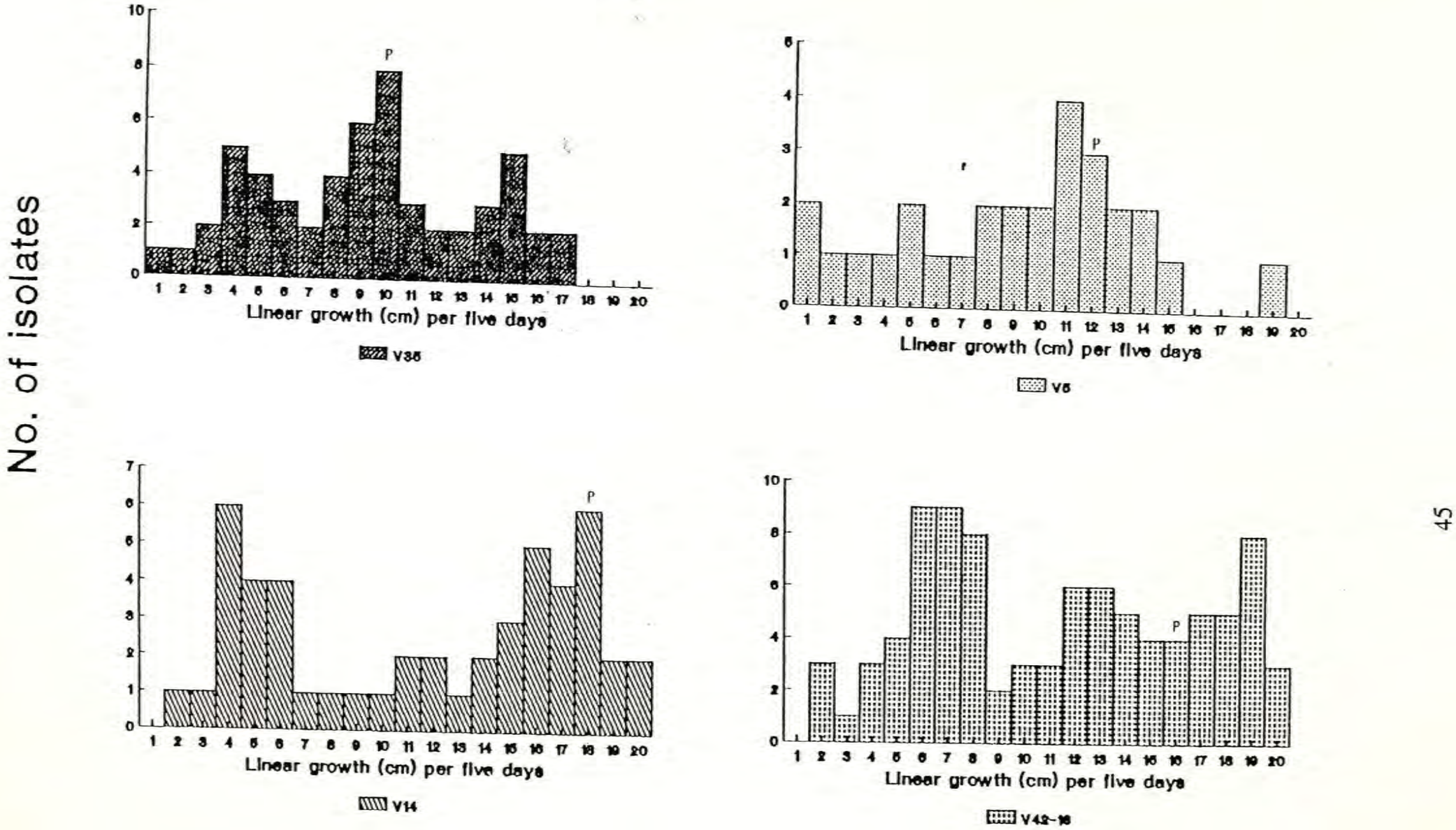


Fig. 3.3.(a) Distribution of linear growth rates in monosporous isolates.

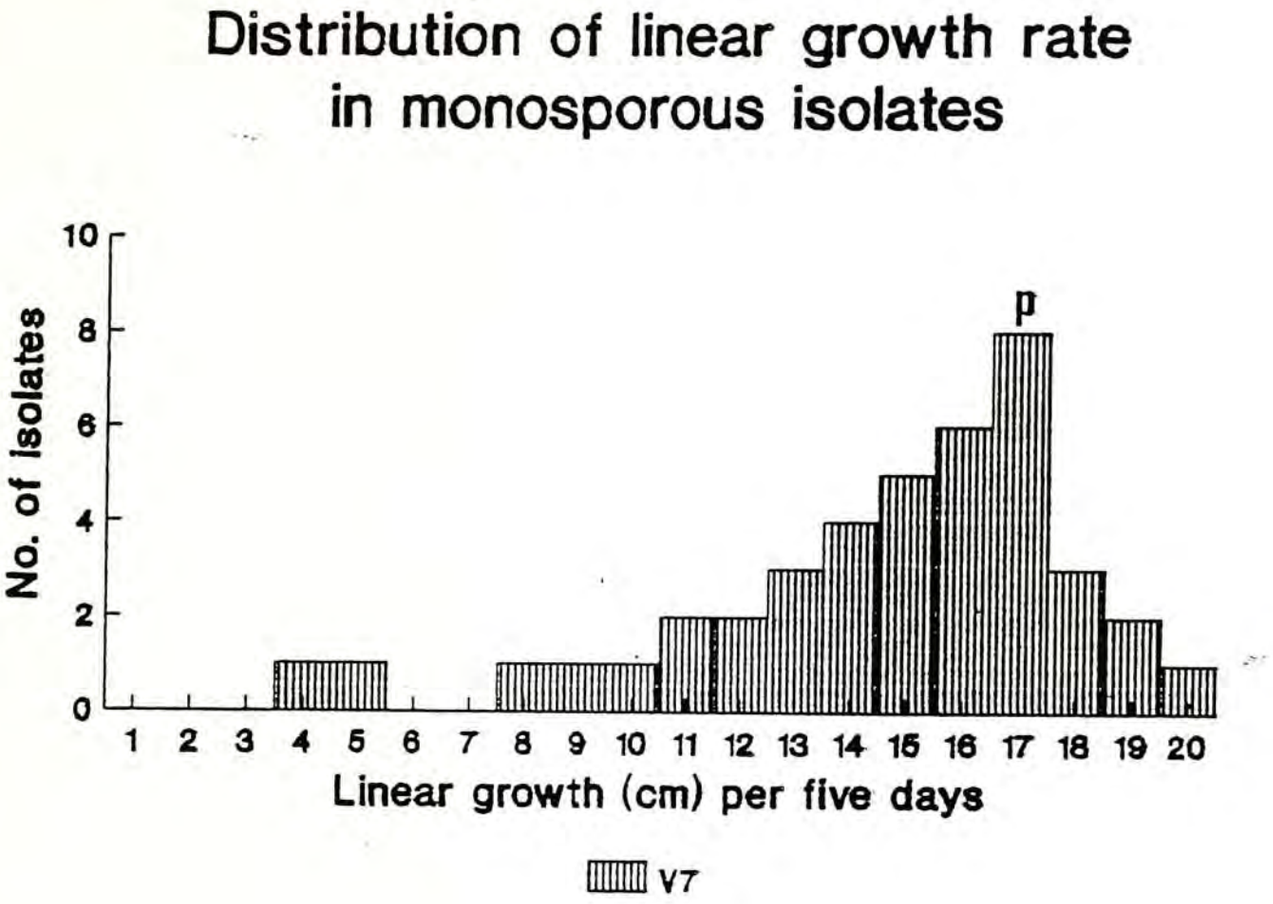


Fig. 3.3. (b) Distribution of linear growth rates in monosporous isolates.

3.3.3. Variations in fertility

The fertility of monosporous isolates has been tested. The results from four different strains are shown in Table 3.4.

Table 3.4. Fertility test of monosporous isolates.

Parental strains	No. of isolates tested	Self–fertile		Self–sterile	
		No.	%	No.	%
V_{42-18}	42	30	71.43	12	28.57
V_{14}	31	19	61.29	12	38.71
V_{35}	30	14	46.67	16	53.33
V_{7}	26	11	42.31	15	57.69

It was found that some isolates were self-fertile but some were self-sterile. However, the ratios of self-fertile to self-sterile were different in different strains. In the strain $V_{42\text{-}18}$, the ratio was 7:3; but in the strain V_{35} and V_7, the ratio was about 1:1.

Moreover, the results in Table 3.5 showed that self-fertile isolates can produce fruiting bodies not only in the open bag condition but also in the special device. Sterile isolates can not produce fruiting body in either methods.

Table 3.5. Fertility test with special device.

Monosporous isolates	Fertility test	
	Open bag method	Special device
$V_{42\text{-}18}$	+ (6, 7)	+ (7, 8)
F–1	+ (6, 8)	+ (9, 11)
F–2	+ (7, 8)	+ (10, 12)
F–3	+ (9, 9)	+ (12, 11)
S–1	–	–
S–2	–	–

+: Fertile isolates; -: Sterile isolates;
The numbers in ( ) illustrate the days of primordia (pinhead) appearance.

Clamp connection is conventionally used in examining for sexual compatibility in Basidiomycetes. However, no clamp connection has been found in *V. volvacea.* Three self-sterile isolates derived from strain $V_{42\text{-}18}$ were "crossed" to see whether they could be stimulated to become "fertile" again, but the results were negative. No fruiting bodies were obtained from each "cross".

In addition, the relationship of gross morphology, fertility and the presence of chlamydospores was analyzed. The results (Table 3.6) show that all the fertile isolates and most of sterile isolates could produce chlamydospores. Production of chlamydospores was not related to the mycelial colonial morphology.

Table 3.6. Gross morphology, presence of chlamydospores in monosporous isolates of *V. volvacea*

Strains	No. of isolates observed	Fertility	Kind of colony		Presence of chlamydospores (%)
			N (%)	A (%)	
V_7	11	F	81.82	18.18	100
	15	S	53.33	46.67	80
V_{14}	19	F	84.21	15.79	100
	12	S	33.33	66.67	83.33
V_{42-18}	30	F	83.33	16.67	100
	12	S	66.67	33.33	66.67

(1) N=normal colony.

(2) A=abnormal colony.

(3) F=self–fertility.

(4) S=self–sterility.

The results of comparison of linear growth rate between fertile and sterile isolates are demonstrated by Table 3.7. Although some sterile isolates grew normally, most of them were usually medium or slow growers.

Table 3.7. Comparison of linear growth rate between fertile and sterile isolates.

Strain	Linear growth rate (cm/5 days)	
	Fertile	Sterile
V_7	16.04 ± 0.30	12.16 ± 0.96 *
V_{14}	15.32 ± 1.01	7.22 ± 0.98 **
V_{35}	12.68 ± 1.04	8.53 ± 1.08 *
V_{42-18}	14.36 ± 0.85	8.01 ± 0.29 **

Values are presented as MEAN ± SEM

Null hypothesis of no difference between fertile and sterile isolates was rejected at $P<0.05$ (*) or $P<0.01$ (**) by Student's t–test.

3.3.4. Comparison of extracellular cellulase activity between fertile and sterile monosporous isolates.

The activity of extracellular cellulase between fertile and sterile monosporous isolates are presented in Fig.3.4. Values are expressed as glucose equivalent (μg/ml) produced per min per ml crude filtrate. The maximum activity of cellulase has been shown to occur about 15 days after inoculation in the parental strain $V_{42\text{-}18}$, fertile isolates F-1, F-2, as well as the sterile strains SN-1 and SN-2. However, the cellulase activities of sterile isolates with abnormal colony morphology are very low.

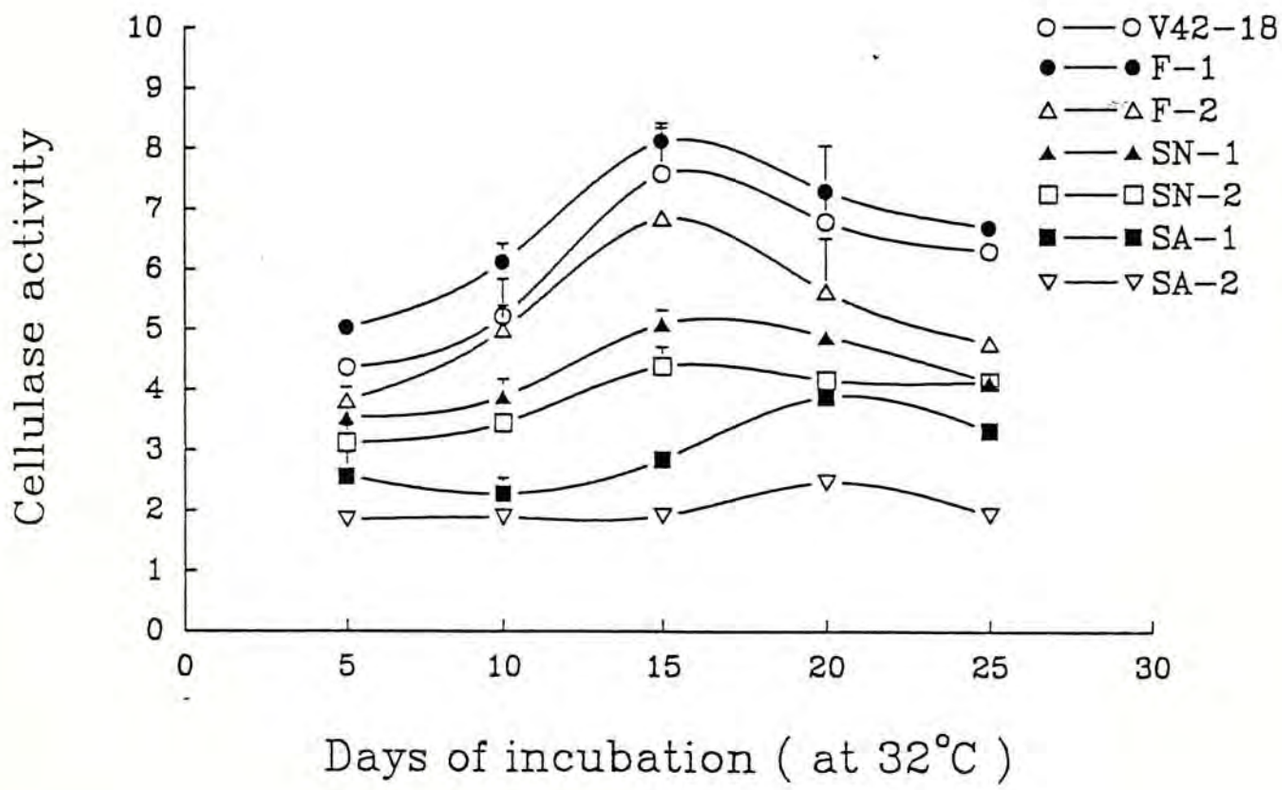


Fig. 3.4. Cellulase activity in relation to incubation period. Values are expressed as glucose equivalent (μg/ml) produced per min per ml crude filtrate.

Fig.3.5 gives the results of mycelial dry weight of the monosporous isolates tested. As in the case of linear growth rate test, some sterile isolates grew normally but some grew slowly when compared with their parental strain (Fig. 3.4).

The relationship between cellulase activity and mycelial dry weight was also studied (Fig.3.7and 3.8). The maximum production of mycelial dry weight was also found to be at 15th day after inoculation. It is similar to that of cellulase.

Comparisons of cellulase activity and mycelial dry weight were also made between fertile and sterile monosporous isolates (Table 3.8 and 3.9).

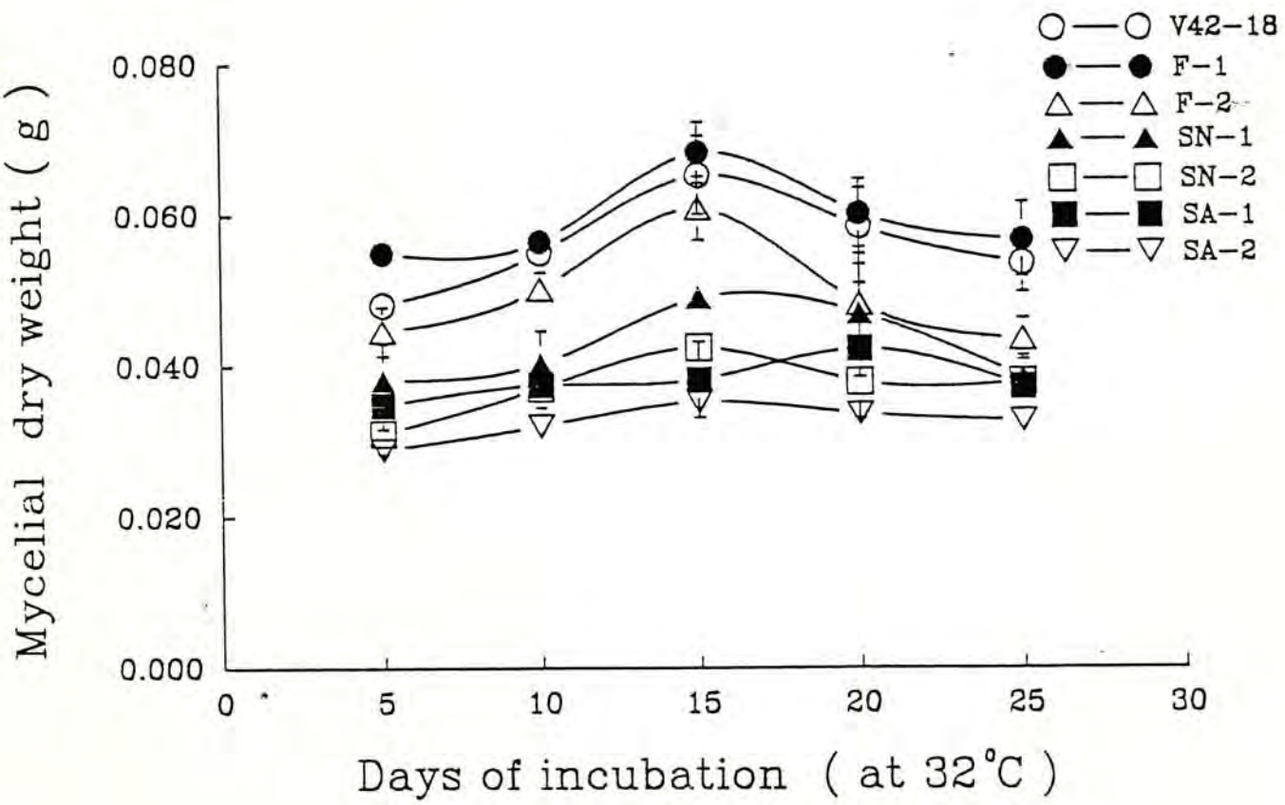


Fig. 3.5. Mycelial dry weight of monosporous isolates tested.

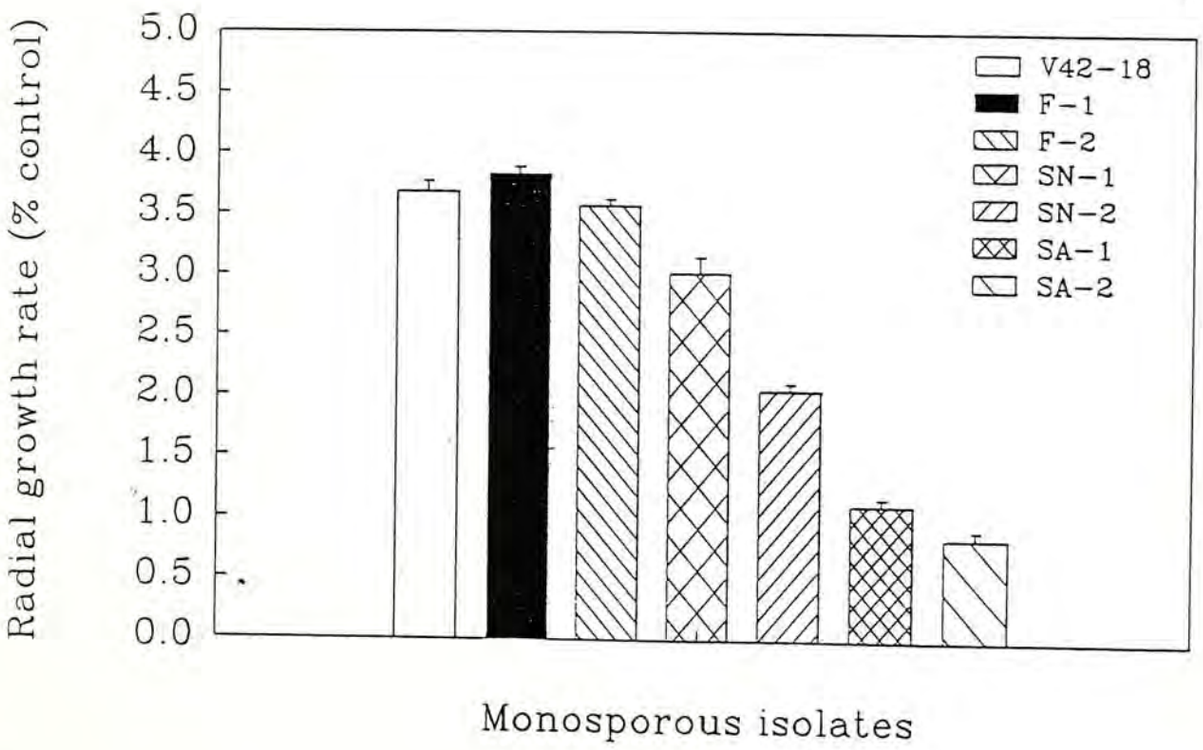


Fig. 3.6. Radial growth rate of monosporous isolates tested.

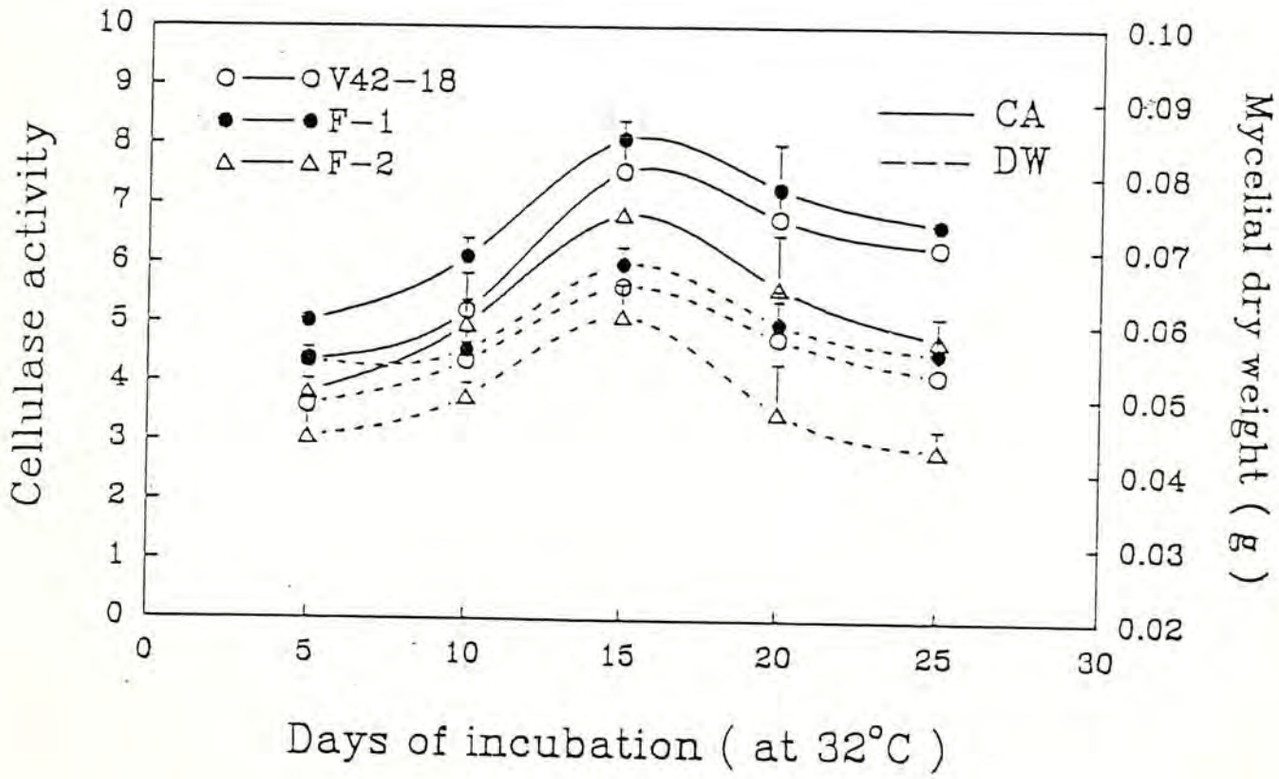


Fig.3.7.Cellulase activity and mycelial dry weight of Self-fertile isolates. Values of cellulase activity are expressed as glucose equivalent (μg/ml) produced per min per ml crude filtrate.

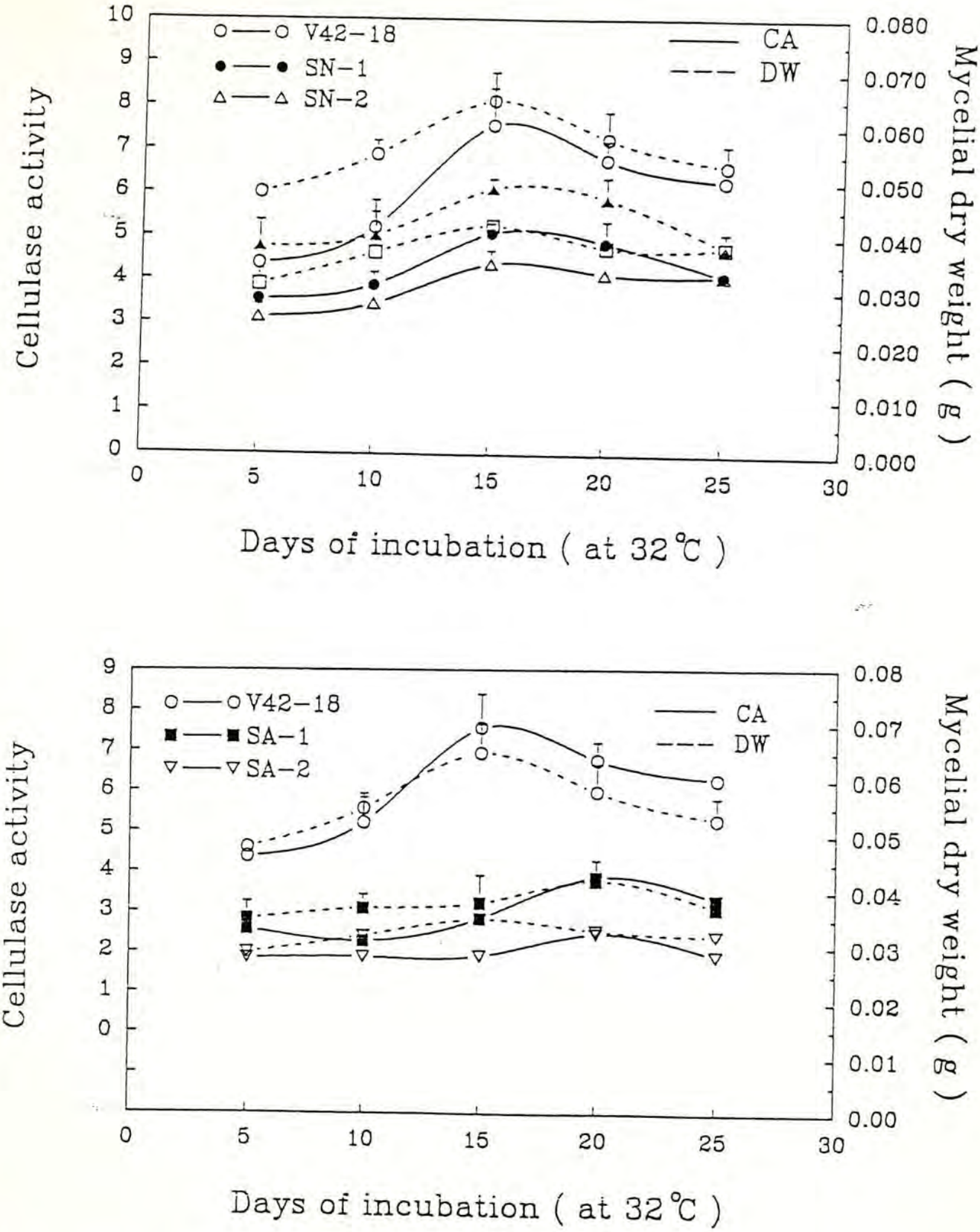


Fig. 3.8. Cellulase activity and mycelial dry weight of self-sterile isolates. Values are expressed as glucose equivalent (μg/ml) produced per min per ml crude filtrate.

Table 3.8. Comparison of cellulase activity between fertile and sterile monosporous isolates.

MI	GPM	SA-2	SA-1	SN-1	SN-2	F-2	V_{42-18}	F-1
SA-2	1.898							
SA-1	2.860							
SN-2	3.837	*						
SN-1	4.293	*	*					
F-2	5.196	*	*	*				
V_{42-18}	6.033	*	*	*	*			
F-1	6.638	*	*	*	*	*		

MI: Monosporous isolates.

GPM: Group means are based on 5 different days of triplicates of each monosporous isolate.

*: Significant difference at P = 0.05 (one way analysis of variance followed by Duncan's multiple range test).

Table 3.9. Comparison of mycelial dry weight between fertile and sterile monosporous isolates.

MI	GPM	SA-2	SN-2	SA-1	SN-1	F-2	V_{42-18}	F-1
SA-2	0.0322							
SN-2	0.0372							
SA-1	0.0378							
SN-1	0.0420	*						
F-2	0.0494	*	*	*				
V42-18	0.0558	*	*	*	*			
F-1	0.0590	*	*	*	*	*		

MI: Monosporous isolates.

GPM: Group means are base on 5 different days of triplicates of each monosporous isolate.

*: Significant difference at P = 0.05 (one way analysis of variance followed by Duncan's multiple range test)

3.4. Discussion

In this study, an attempt was made to find out whether there were variations in monosporous isolates. In addition, a special device was set up to conduct their self-fertility. The experimental data showed that there was a wide range of variation in monosporous isolates even though they were derived from one sporocarp. These results were consistent with those previously reported by Li and Chang (1979) and Chang *et al.*, (1981).

3.4.1. Study on the colonial morphology of monosporous isolates

Colonial morphology is an apparent character to be observed and compared. With reference to Fig.3.2 and Table 3.3, "normal" and "abnormal" colony morphology were found in monosporous isolates, but the ratio varied with different strains.

As pointed out by Li and Chang (1979), expression of morphological characters depended not only upon the genotype, but also to a considerable extent upon external factors. In this study, the role of environmental factors was not taken into account since all cultures were grown under uniform and identical conditions as far as possible. Nevertheless, the occurrence of "normal" and "abnormal" colony morphology indicates the variations of culture type in monosporous isolates.

3.4.2. Study on the growth rate of monosporous isolates

Growth is a complex process with many different component processes depending on the organisms and the culture conditions. Therefore, a precise definition of growth that is universally applicable cannot be made. Griffin (1981) has pointed out that growth can be defined according to the criteria for measurement used in the experiment at hand. Thus choice of techniques becomes critical, and understanding their limitations is very important in interpreting the results.

A simple method of measuring fungal mycelial growth is by determining the increase in linear dimension on a solidified medium. Five-day growth was measured because the mycelia were within the growth phase during this period. In addition, the fastest growers nearly attended the maximal length of the growth tube. However, there is limitation in this measuring method since mycelia usually grow radially or in all directions. The results from mycelial dry weight test (Fig.3.5) and radial growth rate (Fig. 3.6) also demonstrate that the differences in growth among monosporous isolates of a single parent are apparent. This variation is similar to that found in linear growth rate test. Isolates might grow normally or abnormally when compared with their parental strain.

3.4.3. Study on the extracellular cellulase activity

Cellulose is a renewable carbon source that is available in large quantities. It is well known that most of edible fungi are capable of growing and fruiting on ligno-cellulosic waste materials such as wood, sawdust, straw and other agricultural plant residues. The cellulolytic enzymes of most edible fungi are considered to be very active (Nakazawa *et al.*, 1974; Ryn and Mandels, 1980; Fan *et al.*, 1980; Knowles *et al.*, 1987).

Although much information is available on the cellulolytic enzymes of fungi (Norkrans, 1957; Rawal *et al.*, 1981; Freer and Detroy, 1982; Doshi *et al.*, 1987; Carder, 1989; Micales and Highley, 1989), cellulase activity of *V. volvacea* which thrives well on cellulosic waste materials has received less attention (Wang, 1981; 1982; 1985). Wang (1981) has shown the ability of mycelium of *V. volvacea* to secrete extracellular cellulolytic enzymes in the culture medium. Furthermore, he demonstrated that the role of cellulases would appear to be similar to that in *Achyla ambisexualis* (Thomas and Mullins, 1967), especially during the early stages of sporocarp development.

The purpose of studying extracellular cellulase is to compare the enzyme activities between self-fertile and self-sterile monosporous isolates. As can be seen from Table 3.8, there is no significant difference of cellulase activity between wild type strain (V_{42-18}) and self-fertile isolates (F-1 and F-2). Although lower cellulase activity can be found among the self-fertile isolates, the cellulase

activity of self-fertile isolate F-2 does not significantly differ from that of self-sterile isolate SN-2, which has normal colony morphology . These results are similar to those derived from mycelial dry weight determination.

Because the sample size is small in this enzyme assay, no definite conclusion can be drawn. Nevertheless, it is still valuable as a reference for further study. Actually, there is considerable variation in cellulase activity among the monosporous isolates although they were derived from the same sporocarp.

3.4.4. Study on the fertility of monosporous isolates

The results of the fertility test showed in Table 3.4 agreed with those described by Chang and Yau (1971), Li and Chang (1979) and Chang *et al.*, (1981). It is a constant phenomenon that self-sterile isolates occur in monosporous isolates of any sporocarp from different strains. Isolates with fast growth rate could be self-fertile or self-sterile. Nevertheless, self-sterile isolates are usually found to be medium or slow growers.

With reference to Table 3.7, there is no strong correlation between the mycelial colony morphology and self-fertility. Moreover, the production of chlamydospores is not related to the colonial morphology.

Great variation in monosporous isolates is not expected to happen in a homothallic species since the basidiospore can give rise directly to a mature fruit

body without any mating. However, the fertility tests of monosporous isolates so far reported (Chang and Yau, 1971; Li and Chang, 1979) were conducted in mushroom houses where it is possible for the presence of spores of *V. volvacea* in the air. Therefore, there might be a different argument or explanation of the results. It is necessary to develop a method to prove the self-fertility of monosporous isolates under strictly isolated conditions.

The main purpose for setting up the special device is to study the self-fertility of single spore mycelium under controlled conditions. In this device, the gas has to pass through the washing-gas bottle before entering the plastic bag. Therefore, contamination by other spores of *V. volvacea* could be prevented. Compared with the double bag method reported by Li (1981), ventilation may be better in this special device.

Results from fertility tests with the special device reported in Table 3.5 demonstrate that self-fertile isolates can produce fruiting bodies not only in the open bag condition but also in the special device. But sterile isolates cannot produce fruiting bodies in either method.

Unlike other species of edible fungi, clamp connections are absent and the hyphal cell is multinucleate in *V. volvacea.* The identification of isolates which are truthfully derived from a single spore is also important although some isolates can fruit in the isolated condition provided by special device. The production of genetically marked strains offers a possible means of resolving this problem.

3.5. Summary

1. Five geographical strains of *V. volvacea* have been used to analyze the characteristics of monosporous isolates.

2. There was a wide range of variation in monosporous isolates even though they were derived from one sporocarp.

3. Variations occurred not only in growth rate but also in colonial morphology, extracellular cellulase activity and fertility.

4. Self-sterile isolates were usually found to be medium or slow growers. However, isolates with fast growth rate could be self-fertile or self-sterile.

5. Production of chlamydospores was not related to the colonial morphology.

6. Results from fertility tests showed that self-fertile isolates can produce fruiting bodies not only in the open bag condition but also in the special device. Sterile isolates cannot produce fruiting bodies in either method.

Chapter four: Genetical studies on variations of *V. volvacea*

4.1. Introduction

Although a considerable variation of monosporous isolates in *V. volvacea* has been found for about twenty years (Chang and Yau, 1971), the mechanism of variation is still not fully understood. It seems paradoxical that a great variation occurs in this homothallic species which can fruit from a single spore without mating.

Some attempts have been made to clarify this puzzling problem, such as morphological and cytological studies, crossing experiments with self-fertile and self-sterile monosporous isolates (Chang and Yau, 1971; Li and Chang, 1979; Chang *et al.*, 1981). Nevertheless, there are still different explanations on the sexuality pattern and variation in *V. volvacea* (Raper, 1978; Elliott and Challen, 1985). On the other hand, clamp connections, common in the fertile mycelia of Basidiomycetes, are not present in this species (Chang, 1972).Because of the multinucleate nature and the absence of clamp connections, the morphology of the heterokaryon would be similar to that of homokaryon in *V. volvacea*. Therefore, it is difficult to prove whether the fertile isolates are derived from the true self-fertile monosporous isolates or have resulted from the cross-mated heterokaryon. *V. volvacea* was suggested to be

perhaps one of the most perplexing of all cultivated mushrooms (Fritsche, 1972; Eger, 1978; Elliott, 1979a; Raper, 1978; Tokimoto and Komatsu, 1978). Evidence of the sexual pattern in *V. volvacea* requires the progeny analysis of stable mutants. This approach was recognized by Raper (1978) as necessary to verify a sexual process and the major events of the life cycle.

In order to achieve this goal and also to explain the possible sources of variations, a genetical approach was carried out. Physical and chemical mutagenesis of basidiospores and mycelial fragments were used in attempts to induce auxotrophic and resistant mutants in various strains. Moreover, the presence of these markers through mycelium into spore-progeny was also traced.

4.2. Materials and methods

4.2.1. Strains and culture condition

Ten wild type strains were used for the selection of mutants. Their sources are listed in Table 4.1.

Mycelia were cultivated on the complete medium (Raper and Miles, 1958) or synthetic potato dextrose agar medium (PDA, Biolife), 42g/l. All cultures were incubated at 32°C for growth and kept at 15 °C for storage.

Table 4.1. *Volvariella volvacea* strains for mutant selection.

Strains	Sources
V_1	Single spore isolate derived from Chang's collection (=H-18, Chang and Yau, 1971)
V_3	Imported from Taiwan, TW 308
V_5	Tissue culture obtained from Thailand
V_6	Imported from Thailand
V_7	Tissue culture from field collection in Pelating Java, Malaysia
V_{14}	Isolated from Tennamaran Estate, Batang Berjuntai, Selangor, West Malaysia
V_{34}	Provided by Mr. Anon Avetragal, Thailand
V_{35}	Hong Kong (= Ta Lung No.4)
V_{39}	Obtained from Institute of Microbiology, Academia Sinica, China
V_{40}	Obtained from Agricultural Research Station, Shangxi, China
$V_{42\text{-}18}$	Single spore isolate derived from strain V_{42} (Hong Kong)

4.2.2. Chemical compounds

Chemical compounds used to induce or select mutants are listed in Table 4.2. All of them can be dissolved in distilled water. Stock solutions were prepared at 200x the required concentrations (Hocart *et al.*, 1990) and stored at 4 °C. For screening resistant mutants, stock solutions of cycloheximide, crystal violet and malachite green were added to autoclaved agar medium at about 50 °C. Concentrations were expressed as μg/ml except those of potassium chlorate and sodium chloride. Since they were tested at much higher levels, the units used were percentage (%, w/v).

Table 4.2 Chemical compounds for mutant induction and selection.

Active ingredient	Chemical Name	Molecular weight	Source
Acriflavine	3,6-Diamino-10-methyl acridinium chloride	259.74	Fluka Ltd.
Crystal violet	N-[4-[bis[4-dimethylamino] phenyl]methylene]-2,5-cyclohexadien-1-ylidine] -N-methylmethanamium chloride	407.99	BDH Chemical Ltd.
Cycloheximide	[3[2(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide	281.4	Sigma Chemical Co. Ltd.
Ethidium bromide	2,7-Diamino-10-ethyl-9-phenyl-phenanthridinium bromide	394.3	Sigma Chemical Co. Ltd.
Malachite green	N-[4-[4-(Dimethylamino) phenyl]phenylmethylene)-2,5-cyclohexadien-1-ylidine]N-methyl-methanaminium Chloride	929.0	Sigma Chemical Co. Ltd.
Potassium chlorate	$KClO_3$	122.55	Merck Ltd.
Sodium chloride	NaCl	58.44	Peking Chemical Works

4.2.3. Determination of sensitivities to growth inhibitor and chemical

To determine the mycelial sensitivity of different strains of *V. volvacea*, 2 mm diameter agar plugs, from the edge of growing colonies, were placed at the centre of PDA plate containing appropriate concentrations of test compounds. Colony diameters were measured in 4 directions after 3-6 days growth at 32°C. The data were used to calculate the radial growth rates. They were expressed as a percentage compared with a control growth in the absence of the fungicides. Similarly, the sensitivity of basidiospores was determined by testing their germination rate on PDA containing different concentrations of tested compounds. Dose response curves were plotted and the minimum concentration which completely inhibited growth or spore germination (MIC) were determined. All the response tests were performed at least in triplicate. Potato dextrose agar (PDA) medium containing 2-5 fold MIC of growth inhibitors was used to select spontaneous or UV-induced resistant mutants.

4.2.4. UV mutagenesis

Both basidiospores and mycelial fragments were used for mutant induction. A suspension of mycelial fragment of 5-day old culture was prepared by homogenization of mycelial agar blocks with suitable amount of sterile water in a Waring blender. In order to obtain the hyphae having only one or a few cells and desired regeneration frequency of mycelial fragment, the suitable homogenization time was first determined, which was about 60 seconds at slow

speed. Basidiospores were soaked in distilled water at 32 °C for 24 hours. Ten ml of the mycelial fragment or basidiospore suspensions were poured onto a sterile petri dish with constant magnetic stirring. These suspensions were then exposed at 22 cm to a short-wave UV lamp at an intensity 4 $\mu w/cm^2$ x 100. At different time intervals, samples of the suspension were taken and suitably diluted before spreading onto the synthetic potato dextrose agar medium. A non-irradiated sample was used as a control. These experiments were carried out in the dark to avoid the occurrence of light repair processes after UV irradiation. The number of colonies formed on the culture samples taken at different irradiation times was counted 5-10 days after incubation at 32 °C. UV survival curves were established by plotting the percentage of survival against the exposure time. UV doses causing 95-99.5% mortality were used in an attempt to induce mutants.

4.2.5. Chemical mutagenesis

Two chemical mutagens, acriflavine and ethidium bromide, were used for mutant induction. Both basidiospores or mycelial fragments were exposed to a range of concentrations of mutagens. Response curves were made by calculating the germination rates of basidiospores or regeneration rates of mycelial fragments after mutagen treatment. The concentration of mutagen causing 50% mortality was expressed as LC_{50} (Median lethal concentration). This concentration of mutagen was used to induce mutants.

4.2.6. Enrichment method for auxotroph

After UV irradiation, basidiospores were suspended in the liquid minimal medium and incubated at 32°C for 48 hours. Median lethal concentration of ethidium bromide (Sigma) was then added. The cultures were also incubated at 32°C for 24 hours. Finally they were washed with distilled water and then plated onto complete medium. Also, filtration through a glass wool column was also employed as enrichment for auxotroph after UV treatment.

4.2.7. Isolation and establishment of resistance markers

Putative mutants were obtained by selection of strains showing spontaneous or UV-induced alteration in response to the growth inhibitors. Tolerance to certain compounds may be due to physiological change instead of to mutation. In order to obtain the stable resistant mutants, all putative mutants were retested after subculturing in the absence of growth inhibitor for over three months. The degree of increased tolerance was determined by examining the radial growth rate in the presence of different concentrations of growth inhibitor. Cross resistance was examined by subculturing all of the resistant mutants obtained onto each of MICs of other growth inhibitors supplemented to PDA, including crystal violet, malachite green and cycloheximide.

4.2.8. Isolation and characterization of auxotrophic mutants

For the induction of auxotrophic mutants, both UV and chemical mutagenesis methods were used, as well as enrichment procedures. After these treatments, all survivals were subcultured to modified mushroom minimal medium (Yoo *et al.*, 1988) consisting of: dextrose, 20g/l; K_2HPO_4, 1g/l; $MgSO_4.7H_2O$, 0.5g/l; KH_2PO_4, 0.46g/l; NH_4NO_3, 1g/l; Nobel agar (Difco), 15g/l or Agarose 6g/l. Auxotrophs are unable to grow on the minimal medium. Because *V. volvacea* can grow when a little nutrient or even only agar or agarose were provided, all putative auxotrophs which were unable or grew poorly on minimal medium were retested with complete medium and minimal medium. Identification of the specific mutations was achieved by determining the competence to grow on 12 screening media described by Holliday (1956).

4.2.9. Fruiting and marker segregation test

To determine fertility, all mutants were subjected to small scale fruiting trials. Preparations of compost bag and the fruiting condition are as described in chapter 3.2.4. Because the mutants grew poorly, substrates were also supplemented with the components of complete medium. The segregation of a mutant marker was investigated by analyzing the mutant progenies to see whether the marker is present or not.

4.3. Results

4.3.1. Effects of UV irradiation on mycelium and spore germination rate

Figures 4.1 and 4.2 demonstrate the effects of UV irradiation on mycelium and basidiospores respectively. These UV survival curves were made from the averages of three independent experiments and the values were expressed as the percentage of control. Mycelial fragment seems to be more sensitive to UV irradiation than basidiospores. In the case of strain $V_{42\text{-}18}$, the UV dose causing 99% mortality of mycelial fragment was about 80 seconds. But in spores, it was about 200 seconds.

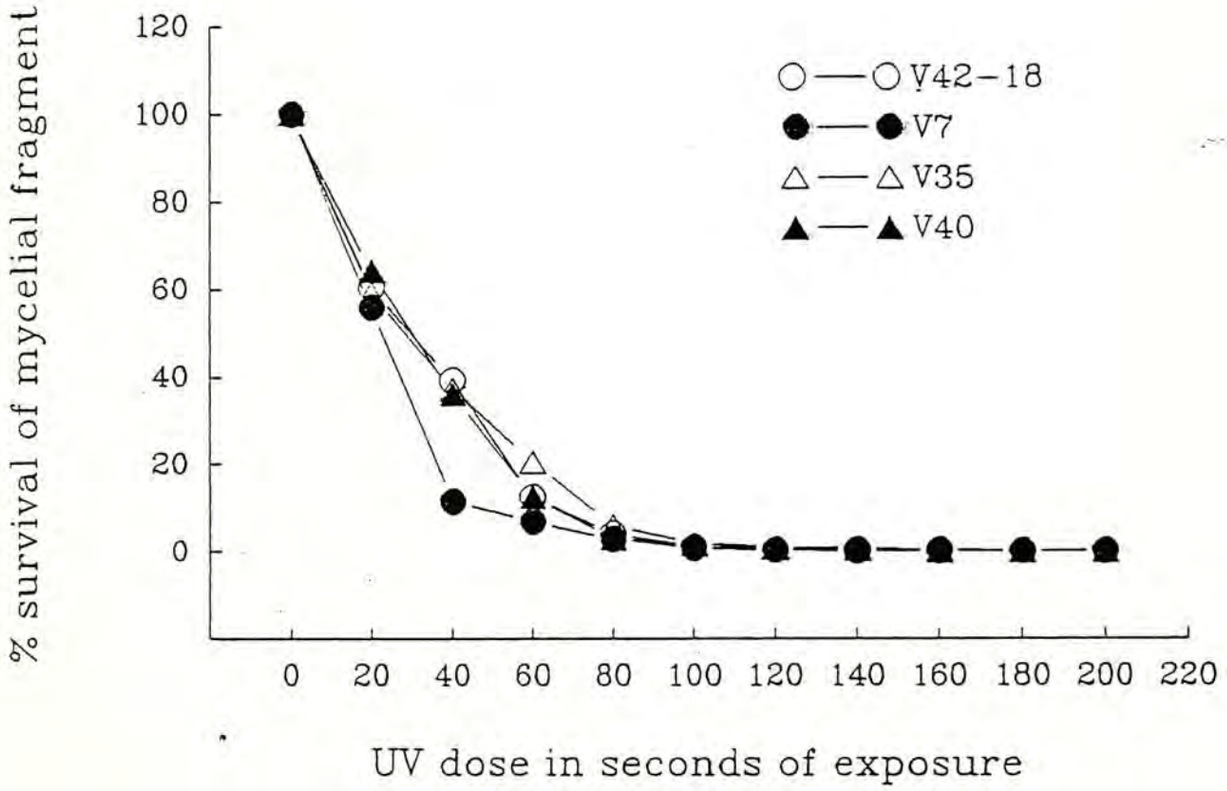


Fig. 4.1. Survival curves showing the effects of UV irradiation on the mycelial fragments of *V. volvacea*. The UV intensity was 4 $\mu w/cm^2$ x 100. The curves were made from the averages of three independent experiments. All the values were expressed as the percentage of control.

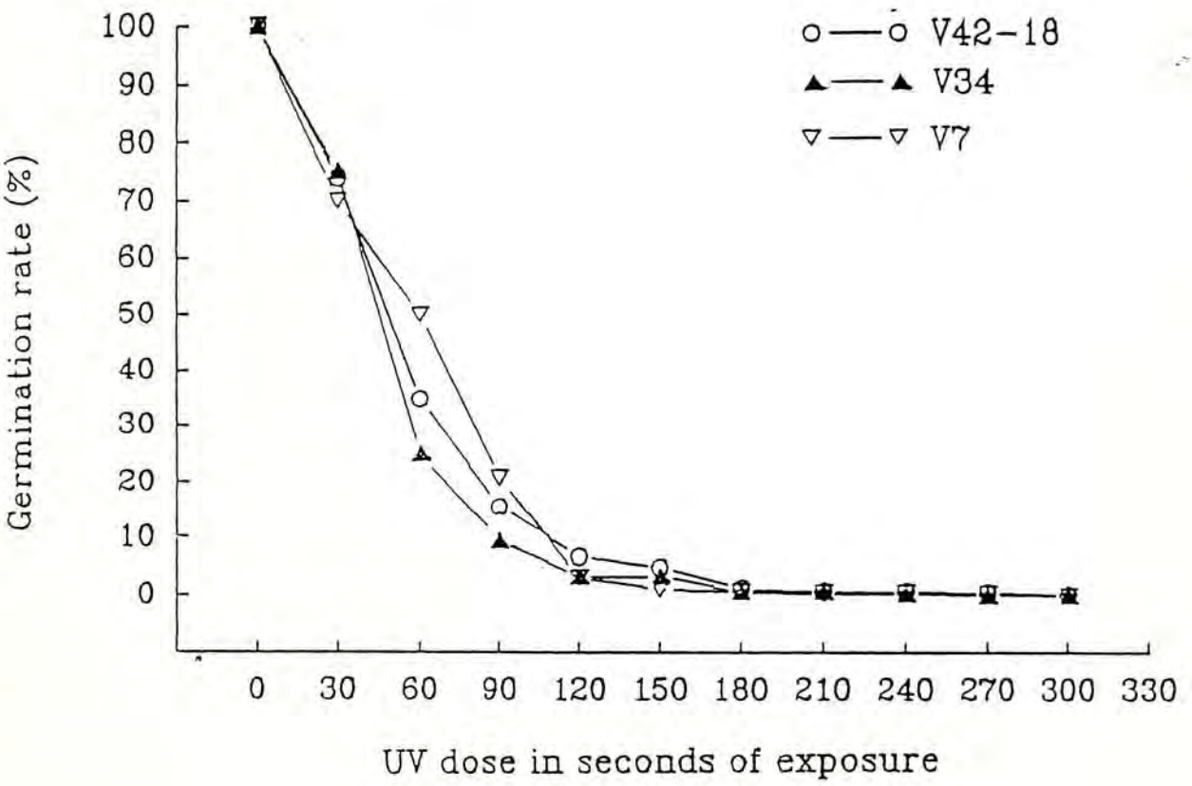


Fig. 4.2. Survival curves showing the effects of UV irradiation on the basidiospores of *V. volvacea* . The UV intensity was 4 $\mu w/cm^2$ x 100. The curves were made from the averages of three independent experiments. All the values were expressed as the percentage of control.

4.3.2. Medium lethal concentrations (LC_{50}) of chemical mutagens

In order to induce mutants by chemical mutagenesis, medium lethal concentrations (LC_{50}) of chemicals must be first estimated. Mortalities were monitored 5 days after mutagen treatment. The criteria for death of basidiospore are lack of germination ability and lysis of the spore wall.

Figures 4.3 and 4.4 show the effects of ethidium bromide and acriflavine on the percentage of mortality of basidiospores respectively. For ethidium bromide, the medium lethal concentration was about 250 μg/ml but for acriflavine, it was about 40 μg/ml. All the data were obtained from three independent experiments and the values were expressed as the percentage of control.

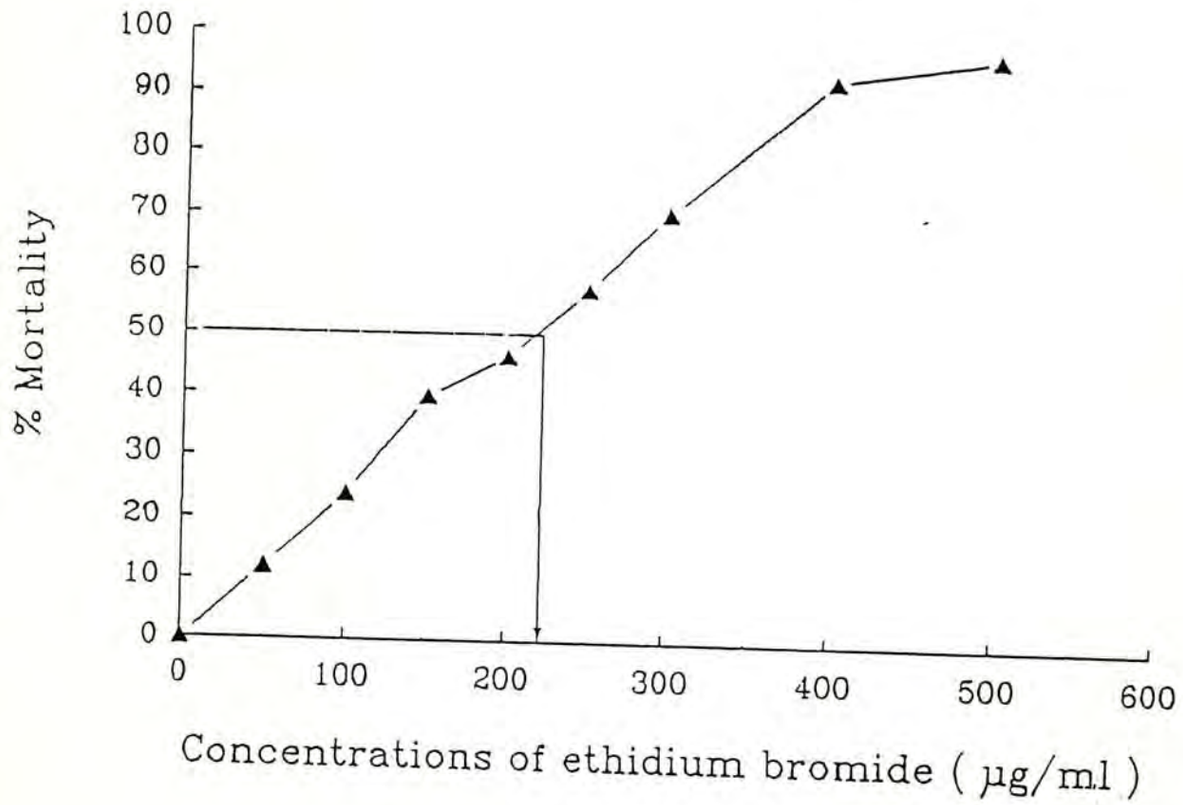


Fig. 4.3. Effects of ethidium bromide on the basidiospores and estimation of medium lethal concentration (LC_{50}).

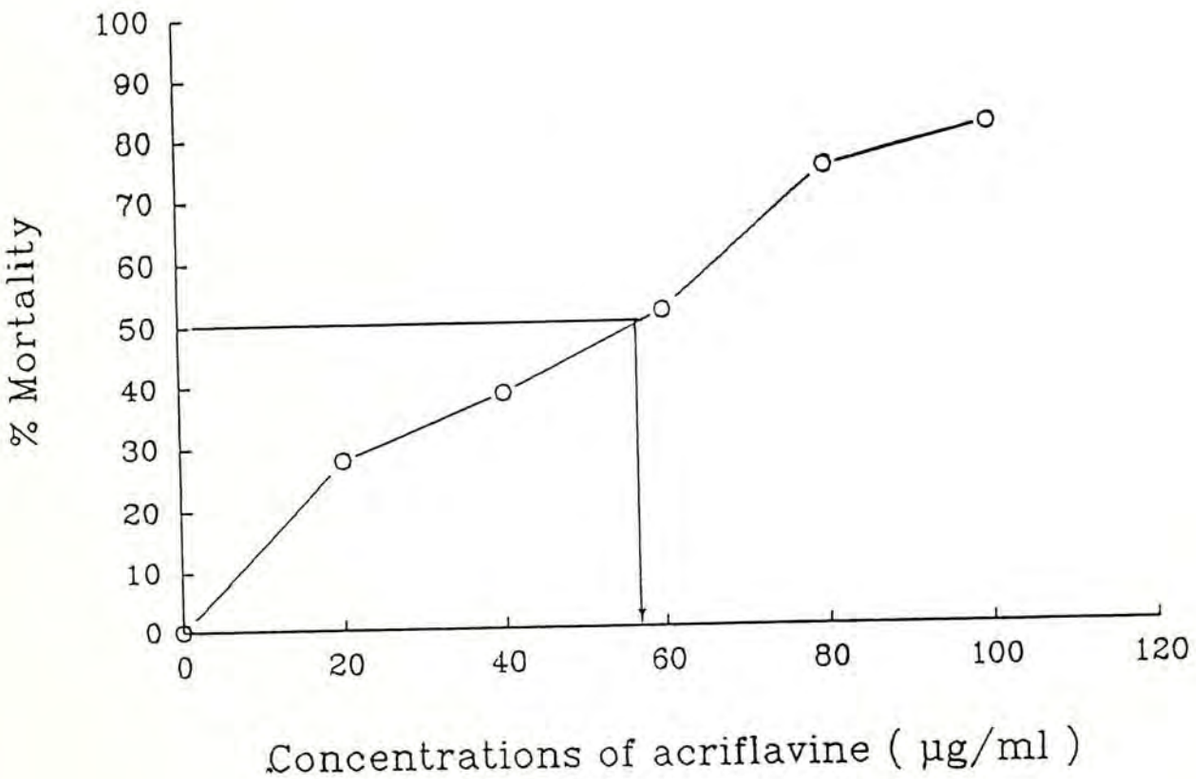
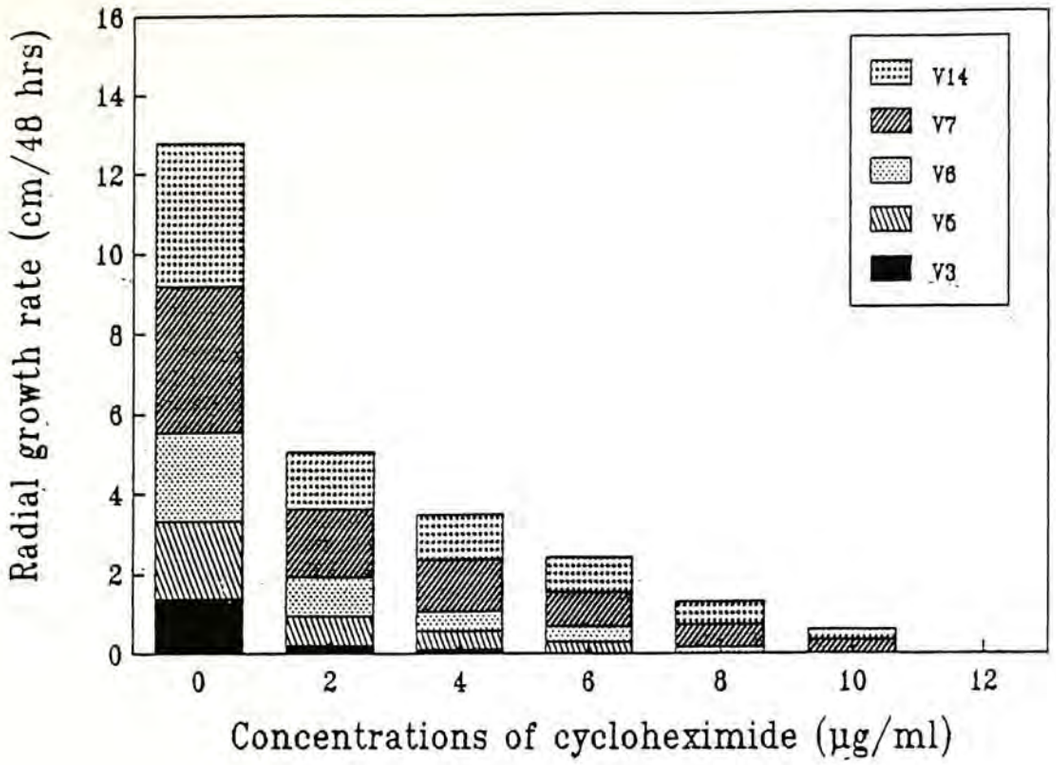


Fig. 4.4. Effect of acriflavine on the basidiospores and estimation of medium lethal concentration (LC_{50}).

4.3.3. Sensitivity of various strains to growth inhibitors and tested compound

In this study, a prerequisite to isolation of resistant mutants is the determination of sensitivity to tested compounds since there may be variations in different strains.The minimum inhibitory concentrations (MICs) of cycloheximide, crystal violet and malachite green were determined. The results are shown in Figs.4.5,4.6 and 4.7 respectively. As the concentrations increase, percentages of inhibition in mycelial growth increase. Moreover, correlation between concentrations of these growth inhibitors and mycelial growth were also analyzed (Tables 4.3, 4.4 and 4.5). All the strains tested were unable to grow on 3 μg/ml crystal violet, 12 μg/ml cycloheximide and 0.5 μg/ml malachite green.

Variations among different strains of *V. volvacea* in their tolerance to sodium chloride and potassium chlorate were investigated. The results are illustrated in Figs.4.10,4.11 and 4.12.



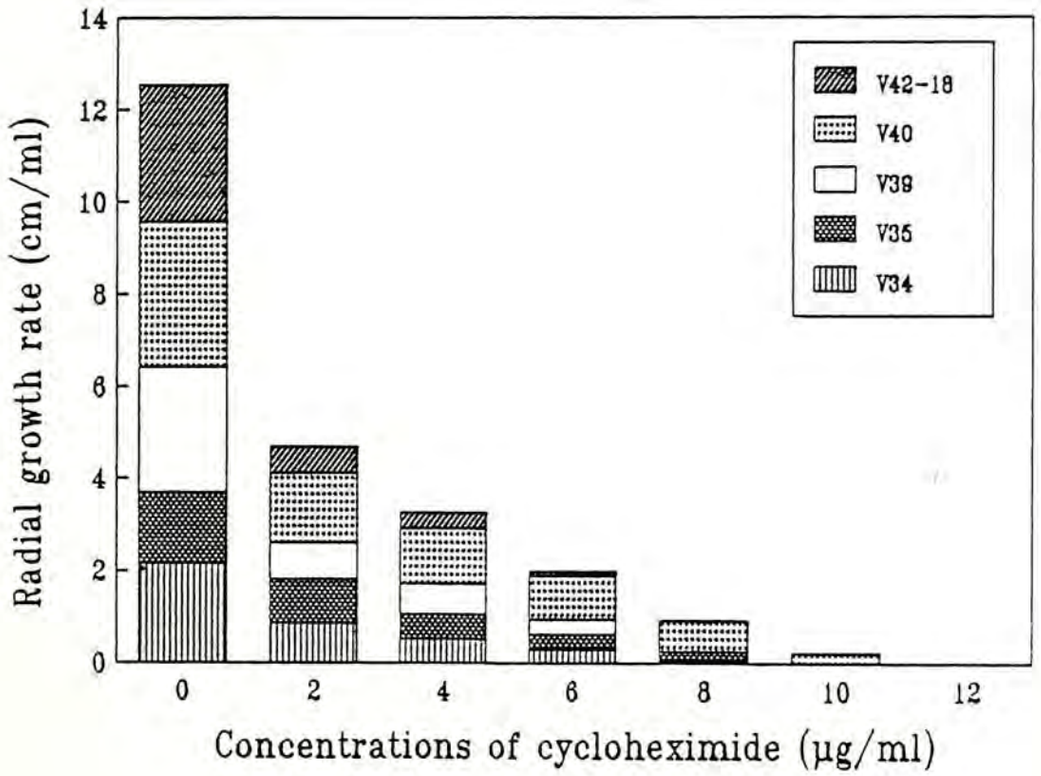
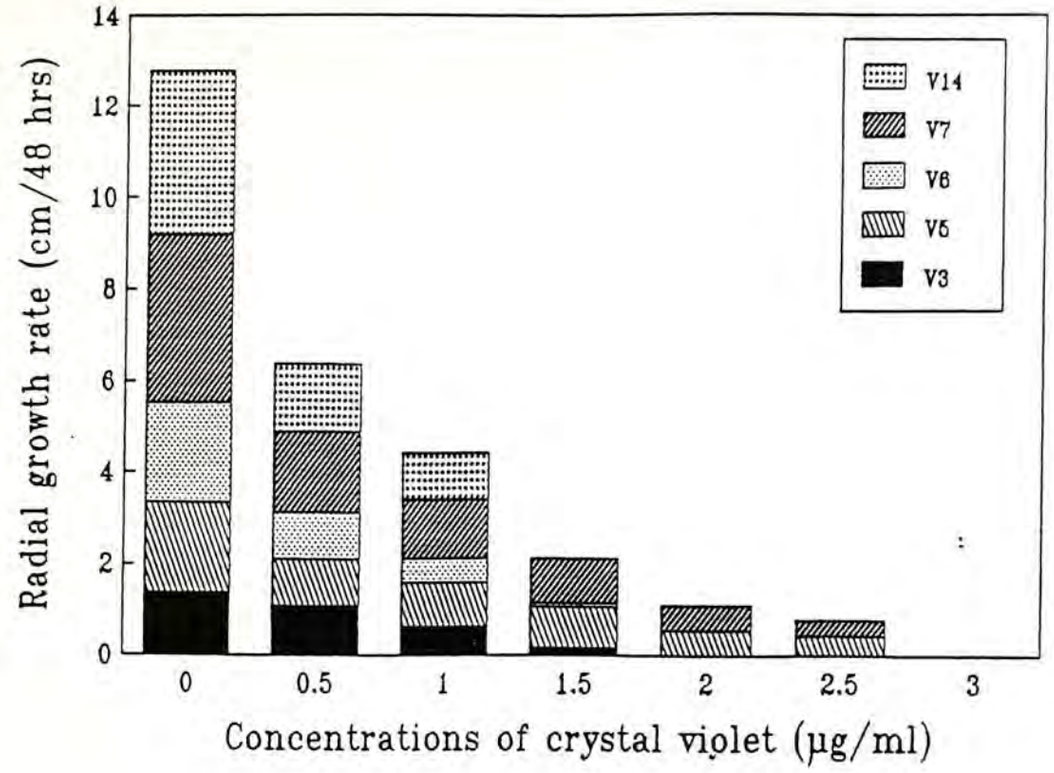


Fig.4.5. Determination of minimum inhibitory concentrations (MICs) of cycloheximide supplemented to PDA for mycelial growth. All values are expressed as the mean of radial growth rate (cm/48hrs) for triplicate samples of three independent experiments.



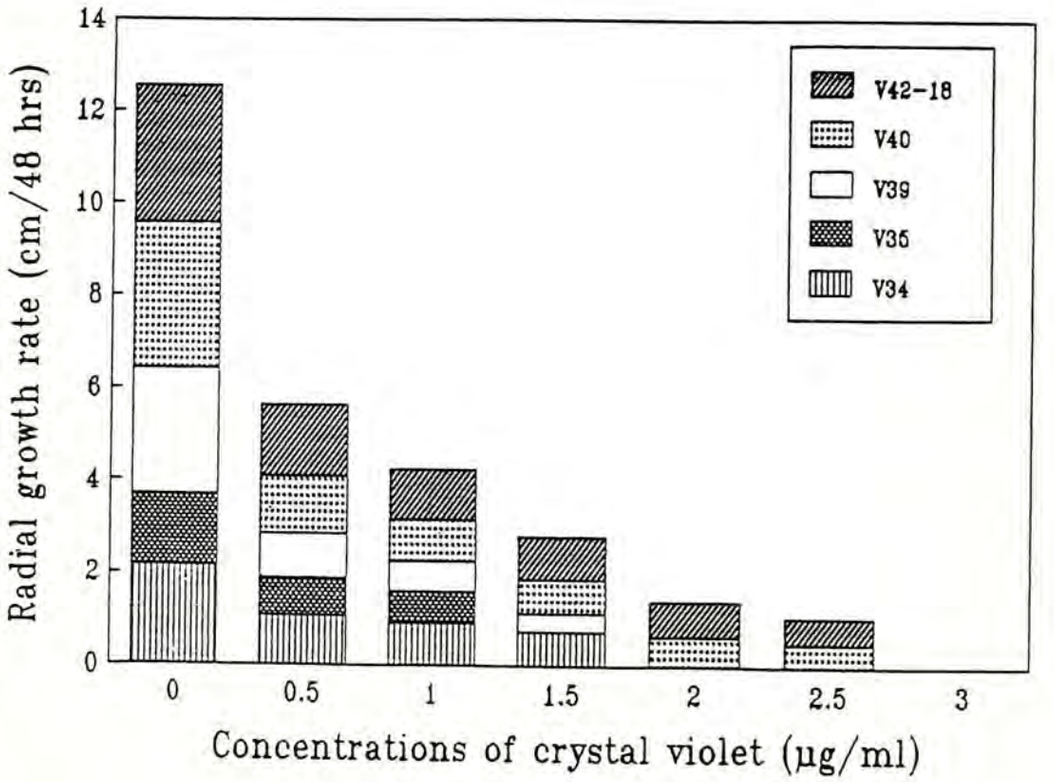


Fig.4.6. Determination of minimum inhibitory concentrations (MICs) of crystal violet supplemented to PDA for mycelial growth. All values are expressed as the mean of radial growth rate (cm/48hrs) for triplicate samples of three independent experiments.

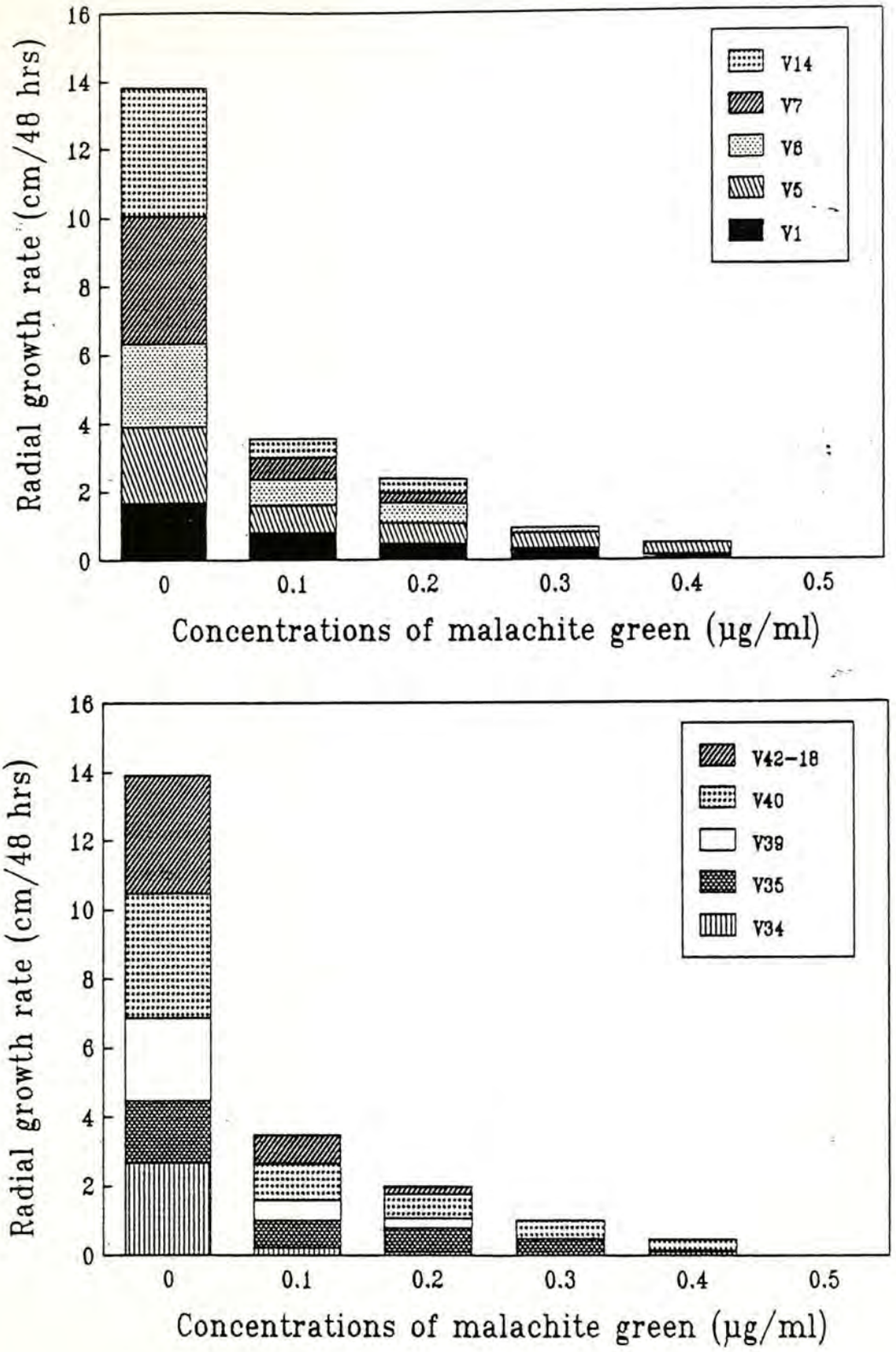


Fig.4.7. Determination of minimum inhibitory concentrations (MICs) of malachite green supplemented to PDA for mycelial growth. All values are expressed as the mean of radial growth rate (cm/48hrs) for triplicate samples of three independent experiments.

Table 4.3. Percentage of inhibition in mycelial growth (cm/48hrs) at different concentrations of cycloheximide.

Strain	Percentage of inhibition in mycelial growth at concentrations (μg/ml)						Correlation coefficients and Regression equations between growth rate (y) and CY conC.(x)	
	2	4	6	8	10	12		
V3	85	92	100	100	100	100	y=0.740-0.083x	(r=-0.71)
V5	62	77	85	100	100	100	y=1.340-0.141x	(r=-0.86)
V6	56	78	83	93	100	100	y=1.535-0.157x	(r=-0.87)
V7	53	64	77	85	100	100	y=2.774-0.260x	(r=-0.91)
V14	61	68	75	84	93	100	y=2.575-0.243x	(r=-0.88)
V34	59	75	86	97	100	100	y=1.496-0.156x	(r=-0.87)
V35	38	65	79	87	100	100	y=1.247-0.123x	(r=-0.94)
V39	71	76	88	100	100	100	y=1.756-0.186x	(r=-0.82)
V40	53	62	78	92	100	100	y=2.430-0.222x	(r=-0.92)
V42-18	79	88	97	100	100	100	y=1.702-0.188x	(r=-0.75)

CY: cycloheximide

Table 4.4. Percentage of inhibition in mycelial growth (cm/48hrs) at different concentrations of crystal violet.

Strain	Percentage of inhibition in mycelial growth at concentrations (μg/ml)						Correlation coefficients and Regression equations between growth rate (y) and CV ConC.(x)	
	0.5	1	1.5	2.0	2.5	3.0		
V3	20	54	87	100	100	100	y=2.790-1.039x	(r=-0.91)
V5	49	50	55	72	78	100	y=1.636-0.534x	(r=-0.94)
V6	53	76	95	100	100	100	y=1.527-0.652x	(r=-0.86)
V14	59	71	100	100	100	100	y=2.448-1.052x	(r=-0.85)
V34	50	57	65	100	100	100	y=1.693-0.684x	(r=-0.92)
V35	46	56	100	100	100	100	y=0.776-0.315x	(r=-0.87)
V39	65	76	85	100	100	100	y=1.828-0.768x	(r=-0.85)
V40	61	72	77	80	84	100	y=2.210-0.794x	(r=-0.85)
V42-18	48	64	69	74	81	100	y=2.340-0.805x	(r=-0.92)

CV: crystal violet

Table 4.5. Percentage of inhibition in mycelial growth (cm/48hrs) at different concentrations of malachite green.

Strain	Percentage of inhibition in mycelial growth at concentrations (μg/ml)					Correlation coefficients and Regression equations between growth rate (y) and MG conC.(x)	
	0.1	0.2	0.3	0.4	0.5		
V1	52	71	79	90	100	y=1.394-3.490x	(r=-0.92)
V5	65	73	80	85	100	y=1.712-4.140x	(r=-0.84)
V6	68	77	93	100	100	y=1.850-5.380x	(r=-0.89)
V7	83	92	100	100	100	y=2.570-8.170x	(r=-0.81)
V14	85	89	100	100	100	y=2.562-8.070x	(r=-0.80)
V34	91	96	100	100	100	y=1.718-5.580x	(r=-0.83)
V35	56	61	72	92	100	y=1.516-3.620x	(r=-0.92)
V39	77	88	100	100	100	y=1.710-5.330x	(r=-0.84)
V40	71	81	85	91	100	y=2.658-7.080x	(r=-0.84)
V42-18	75	93	100	100	100	y=2.450-7.730x	(r=-0.84)

MG: malachite green

In addition, studies on the effects of these growth inhibitors on the spore germination rate were also carried out (Figs.4.8 and 4.9).

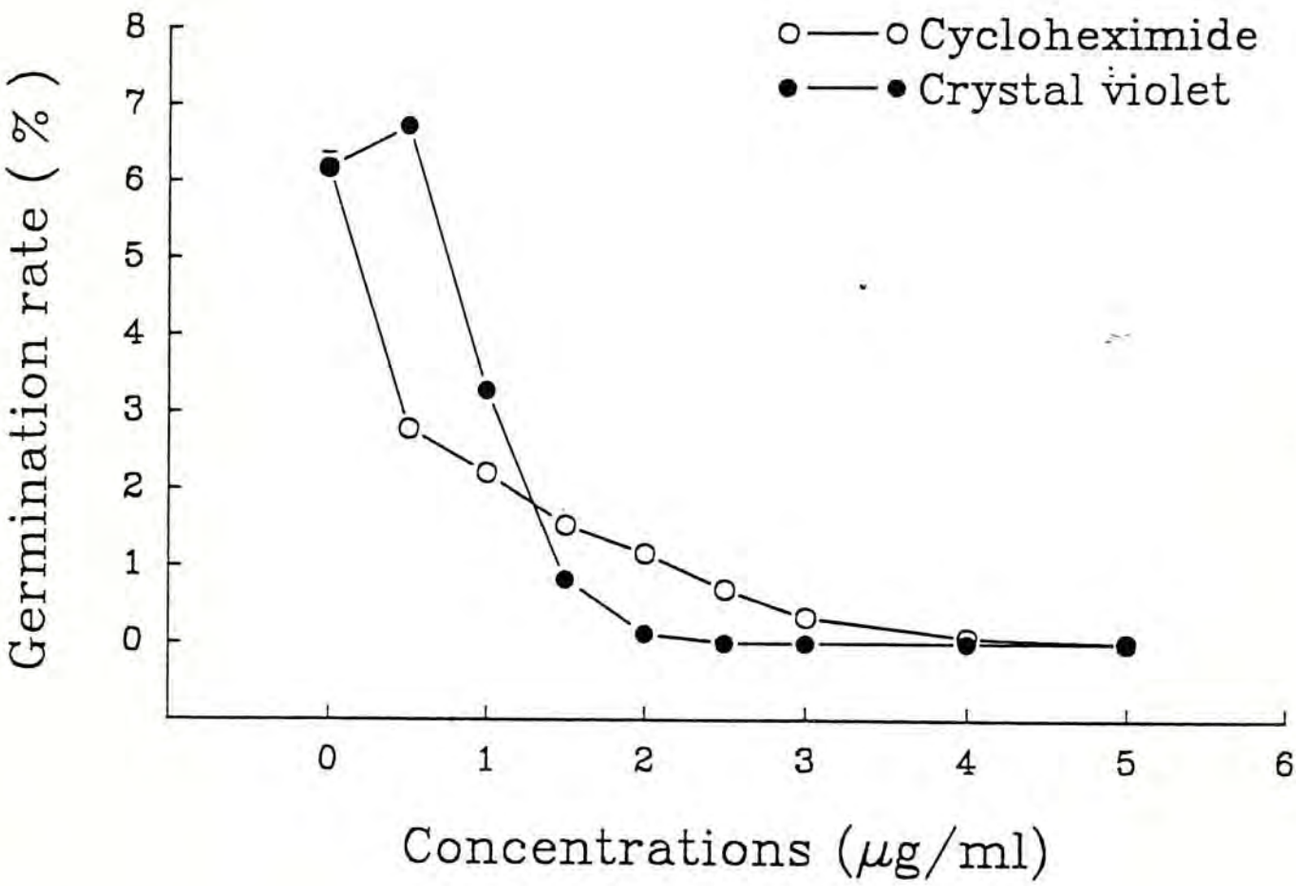


Fig. 4.8. Effects of cycloheximide and crystal violet on the germination rate of basidiospores. Results were obtained from the triplicate samples of three independent experiments. Each point represents the mean ± standard error.

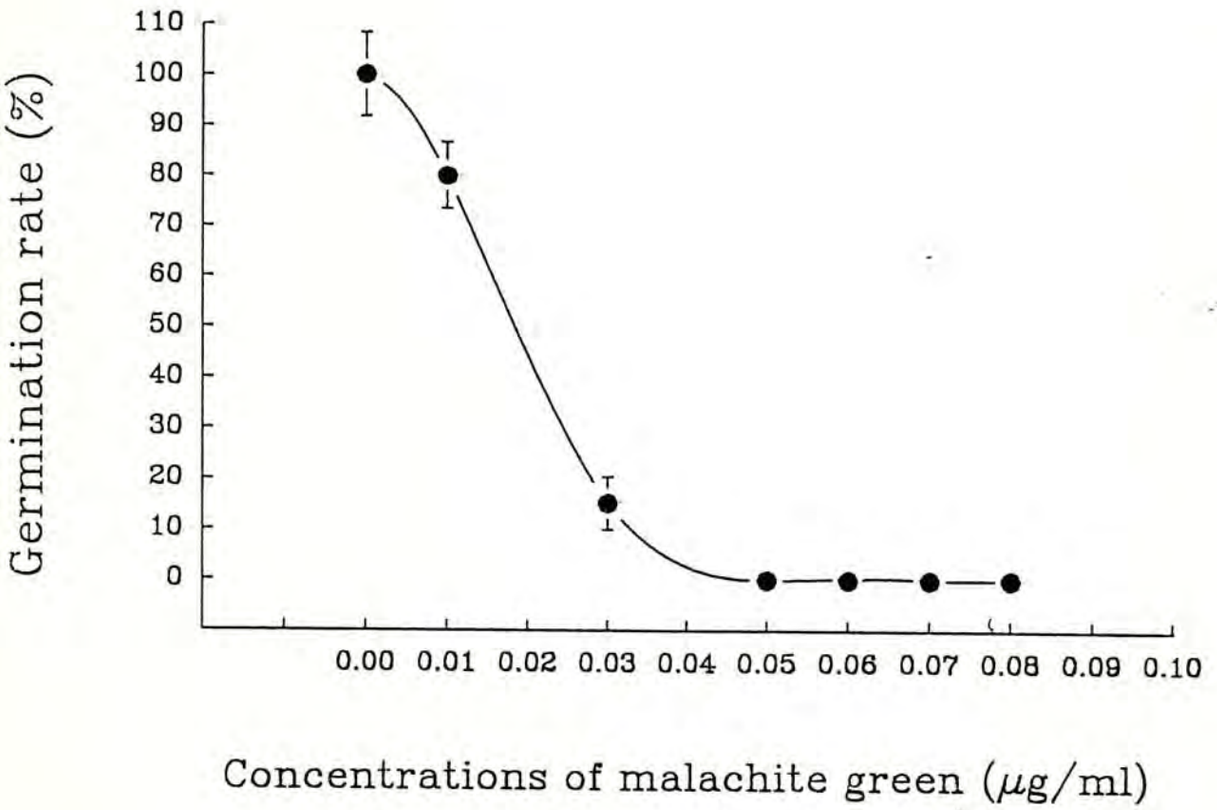


Fig. 4.9. Effects of malachite green on the germination rate of basidiospores. Results were obtained from the triplicate samples of three independent experiments. Each point represents the mean ± standard error.

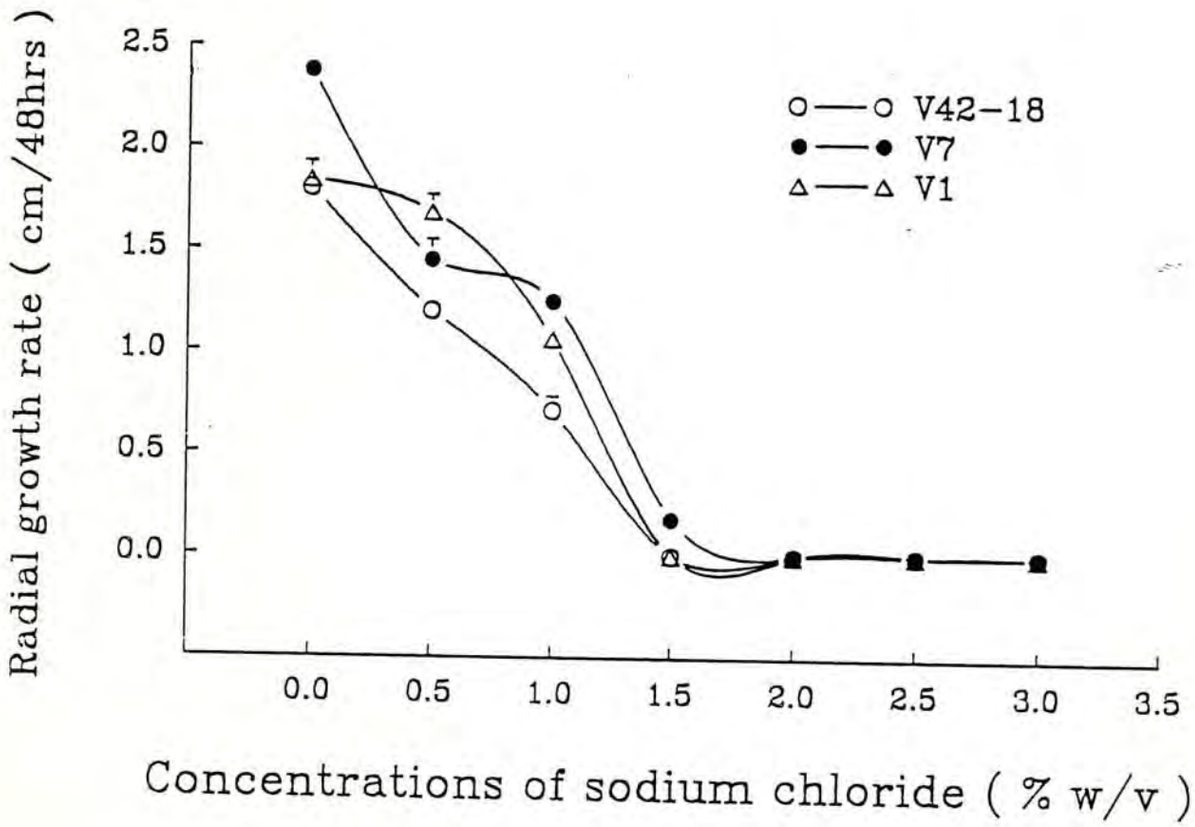


Fig. 4.10. Radial growth rate among different strains of *V. volvacea* to sodium chloride.

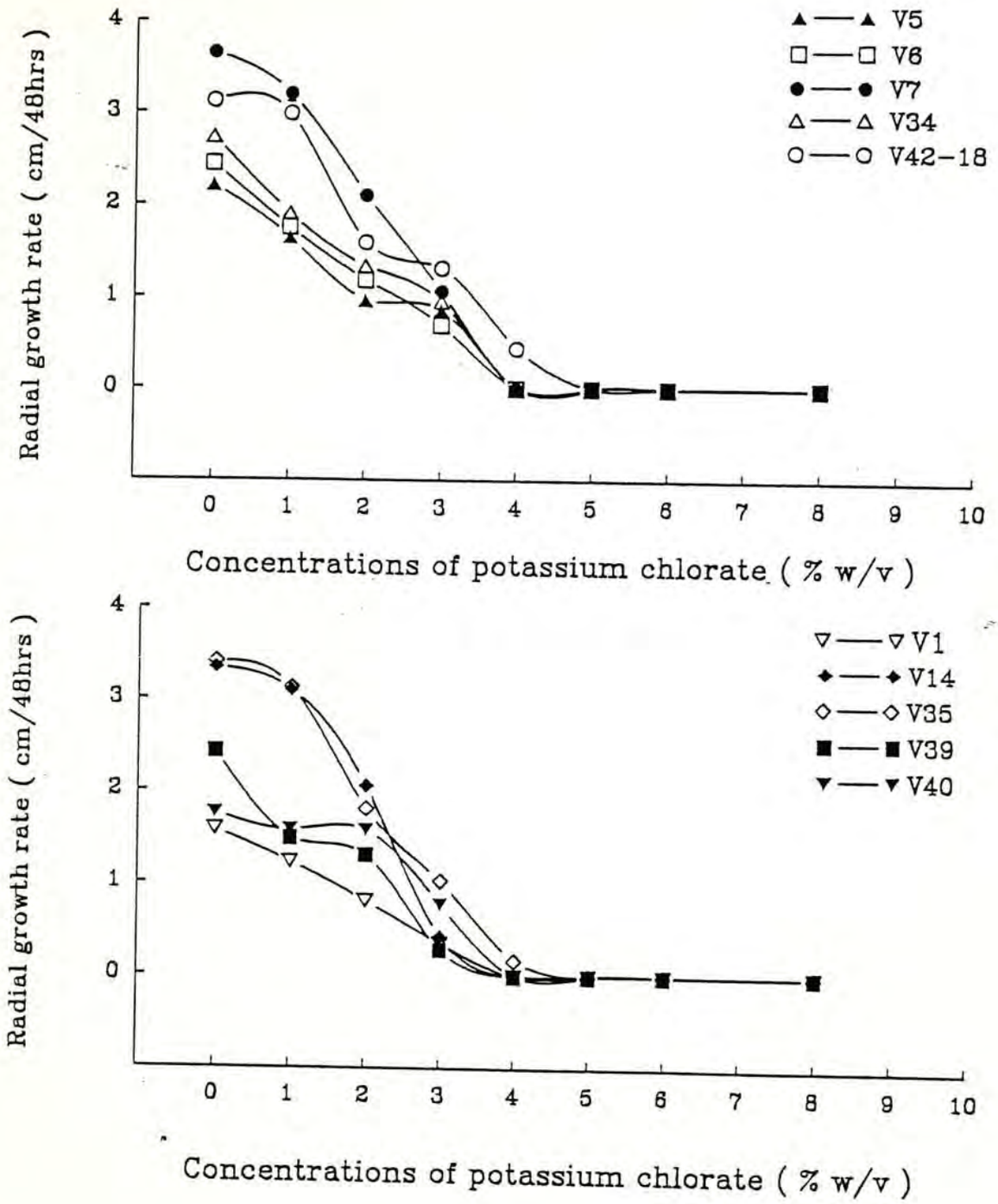


Fig. 4.11. Radial growth rate among different strains of *V. volvacea* to potassium chlorate.

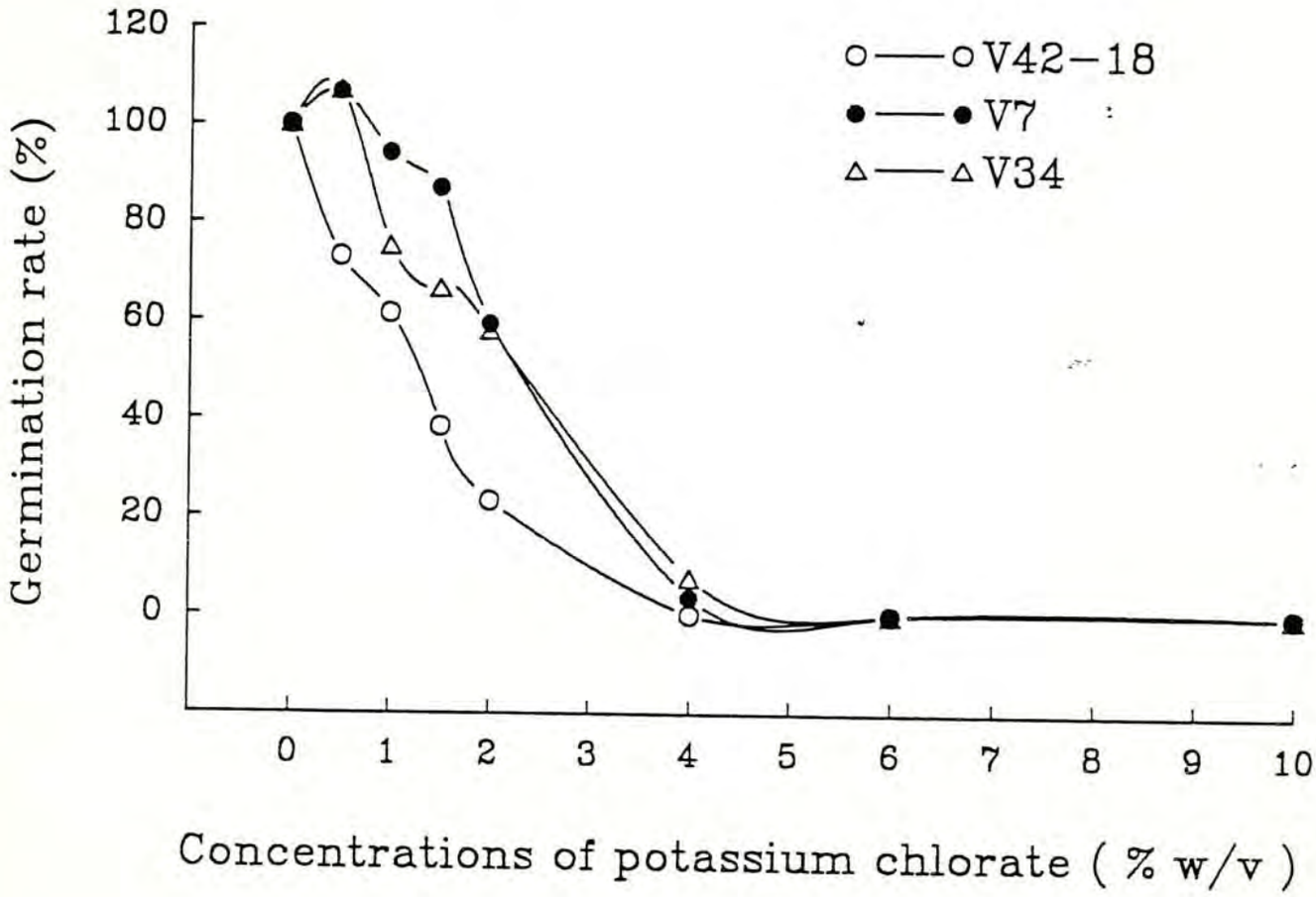


Fig. 4.12. Effect of potassium chlorate on the germination rate of basidiospores.

4.3.4. Identification and characterization of resistant mutants

In the present study, several resistant mutants were obtained. Their parental strain, resistant type and isolation method are summarized in Table 4.6. Stable cycloheximide resistant isolates were obtained in two of the strains, $V_{42\text{-}18}$ and V_7, and these isolates are numbered and designated as *V-cyR* and *V7-cyR* respectively. Crystal violet resistance was obtained only in $V_{42\text{-}18}$, which was named as *V-cvR* . Efforts to induce crystal violet resistance in other strains failed. Malachite green resistant isolates were obtained from $V_{42\text{-}18}$, V_7 and V14, and named *V-mgR*, V_7*-mgR* and V_{14}*-mgR,* respectively, while potassium chlorate and sodium chloride resistant isolates could not be obtained in any of the strains.

Table 4.6. Types of resistant mutants of *V. volvacea*.

Growth inhibitors	Strains screened	No. of putative mutants	No. of stable mutants	Source	Mutant code	Mutant induction
Cyclo-heximide	V_{42-18}	38	4	SP,MF	*V-cyR*	UV,SM
	V_7	21	2	SP,MF	*V7-cyR*	UV
	V_{14}	14	1	MF	*V14-cyR*	UV
	V_5	8	0	SP, MF		
	V_{40}	5	0	MF		
	V_{35}	3	0	MF		
Crystal violet	V_{42-18}	15	3	SP,MF	*V-cvR*	UV
	V_7	6	0	SP,MF		
Malachite green	V_{42-18}	3	1	SP	*V-mgR*	UV
	V_7	3	1	MF	*V7-mgR*	UV
	V_{14}	5	2	MF	*V14-mgR1*	UV
				SP	*V14-mgR2*	SM
	V_1	2	0	MF		
	V_{34}	2	0	SP		
Sodium chloride	V_7	13	0	MF		
	V_5	11	0	MF		
Potassium chlorate	V_7	24	0	SP,MF		
	V_5	26	0	SP,MF		
Total		199	14			

SP: Spore
MF: Mycelial fragment
UV: UV induced mutant
SM: Spontaneous mutant

Two spontaneous cycloheximide resistant mutants from strain V_{42-18} and one spontaneous malachite green resistant mutant from strain V_{14} were obtained at a rate of 2 X 10^{-9} and 5 x10^{-9} respectively. The frequency of recovery of resistant mutants after UV irradiation was much higher, ranging from about 2 X 10^{-5} to 10^{-6}. However, spontaneous cycloheximide resistant mutants have a lower level MIC of cycloheximide when compared with those UV induced (Table 4.7). Three cycloheximide resistant mutants have a characteristic flat morphology on PDA, whereas their parental strain produces abundant aerial mycelium.

Radial growth rate of cycloheximide resistant mutants on different concentrations of cycloheximide is illustrated in Fig.4.13. All mutants showed enhanced tolerance to cycloheximide when compared with the parental strain.

Table 4.7. Minimum inhibitory concentrations among cycloheximide resistant mutants and their parental strains.

Wild type	Mutant	MIC (μg/ml)	No. of strain
$V_{42\text{-}18}$		6	
	V-cyR (SM)	15	1
	V-cyR (SM)	20	1
	V-cyR (UV)	30	2
V_7		10	
	V7-cyR (UV)	40	2

MIC: minimum inhibitory concentration
SM: spontaneous mutant
UV-induced mutant

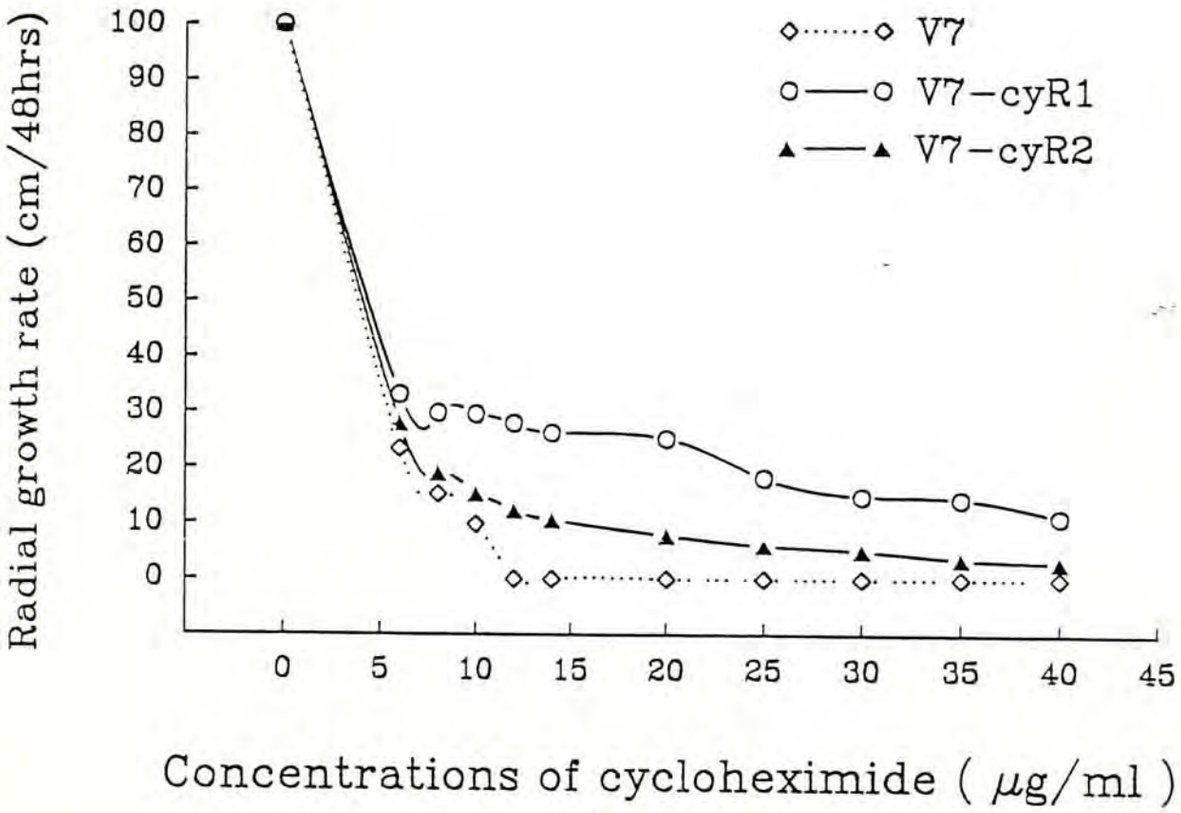


Fig. 4.13. Radial growth rate of cycloheximide resistant mutants and their wild type sensitive strain V_7 in different concentrations of cycloheximide supplemented to PDA.

Fig.4.14 shows the radial growth rate of crystal violet resistant mutants on different concentrations of crystal violet supplemented to PDA. Moreover, cross resistance of resistant mutants have been studied. It was found that crystal violet resistant mutants were generally resistant to malachite green and vice versa (Figs. 4.15 - 4.21). However, cycloheximide resistant mutants were not resistant to either crystal violet or malachite green. They failed to grow on PDA containing MICs of these growth inhibitors.

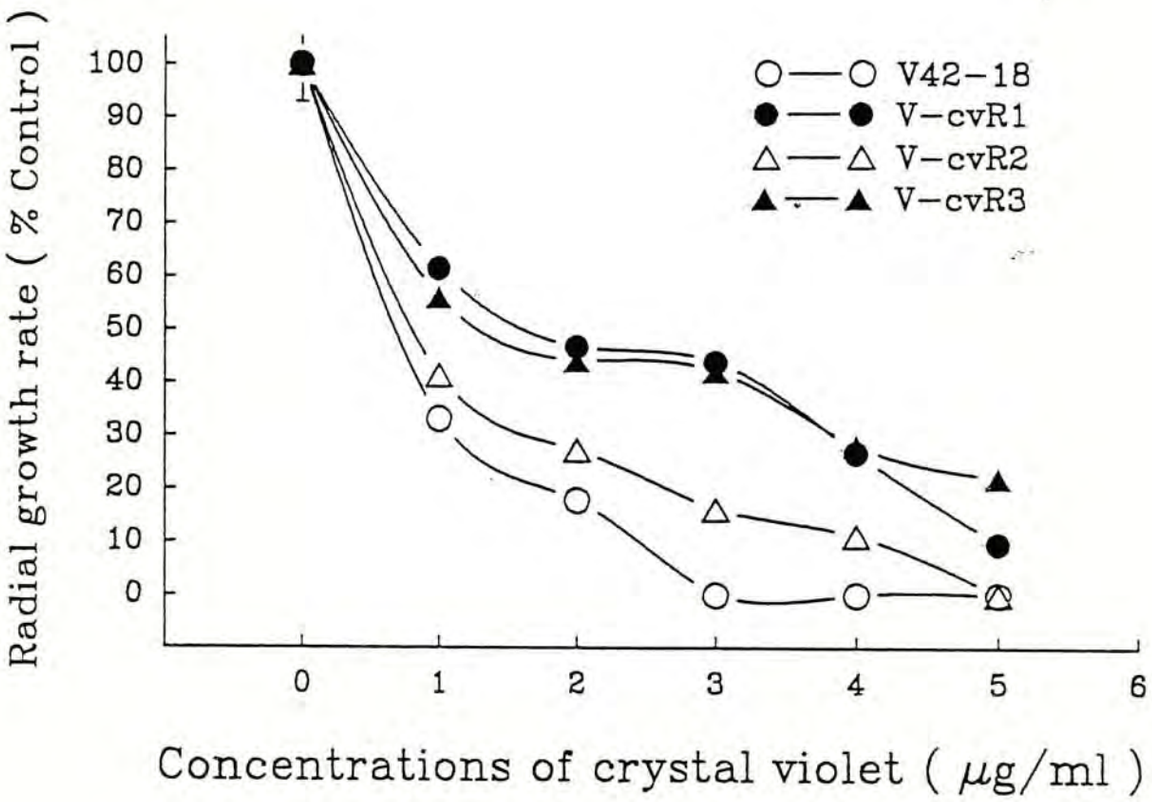


Fig. 4.14. Radial growth rate of crystal violet resistant mutants and their wild type sensitive strain $V_{42\text{-}18}$ in different concentrations of crystal violet supplemented to PDA.

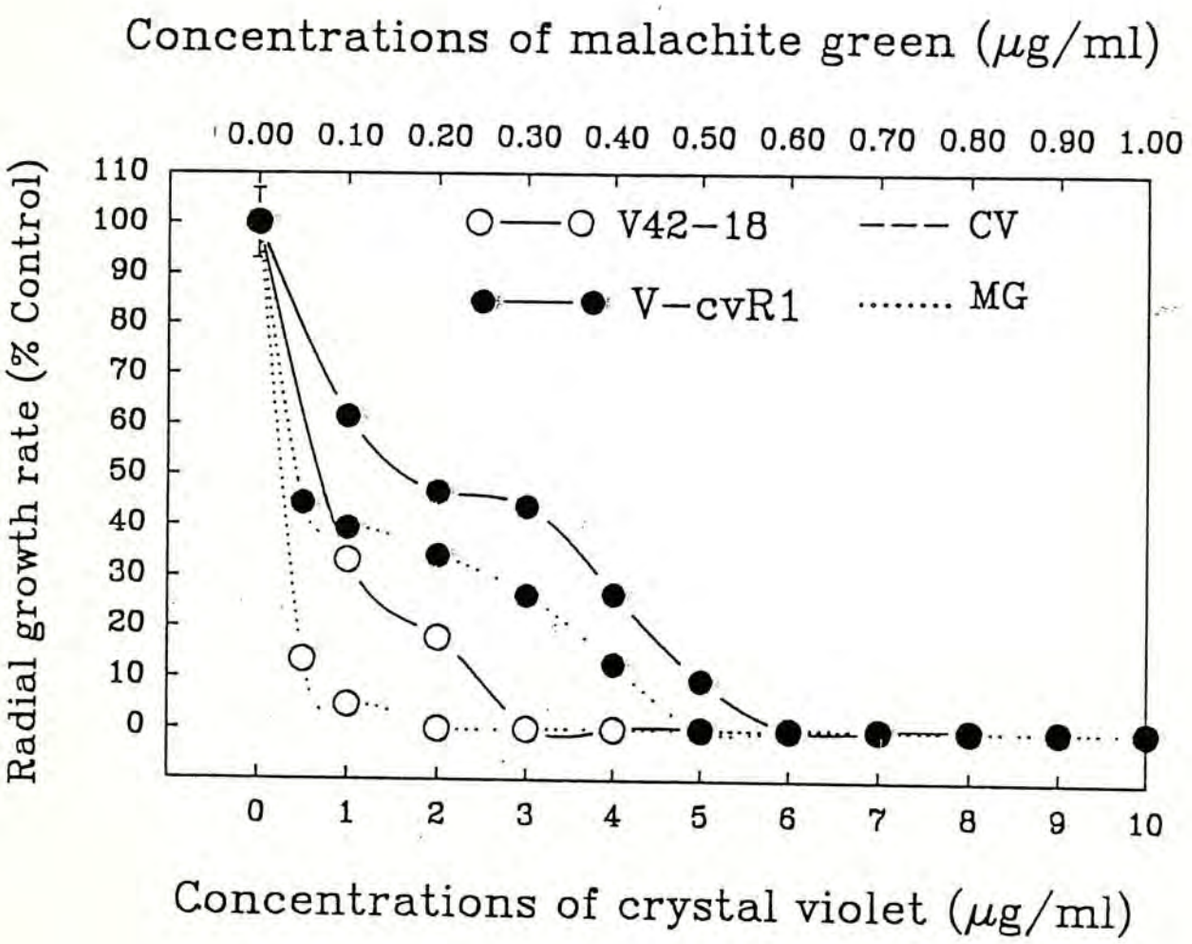


Fig. 4.15. Cross resistance of crystal violet resistant mutant *V-cvR1* and its wild type sensitive strain $V_{42\text{-}18}$ to malachite green supplemented to PDA.

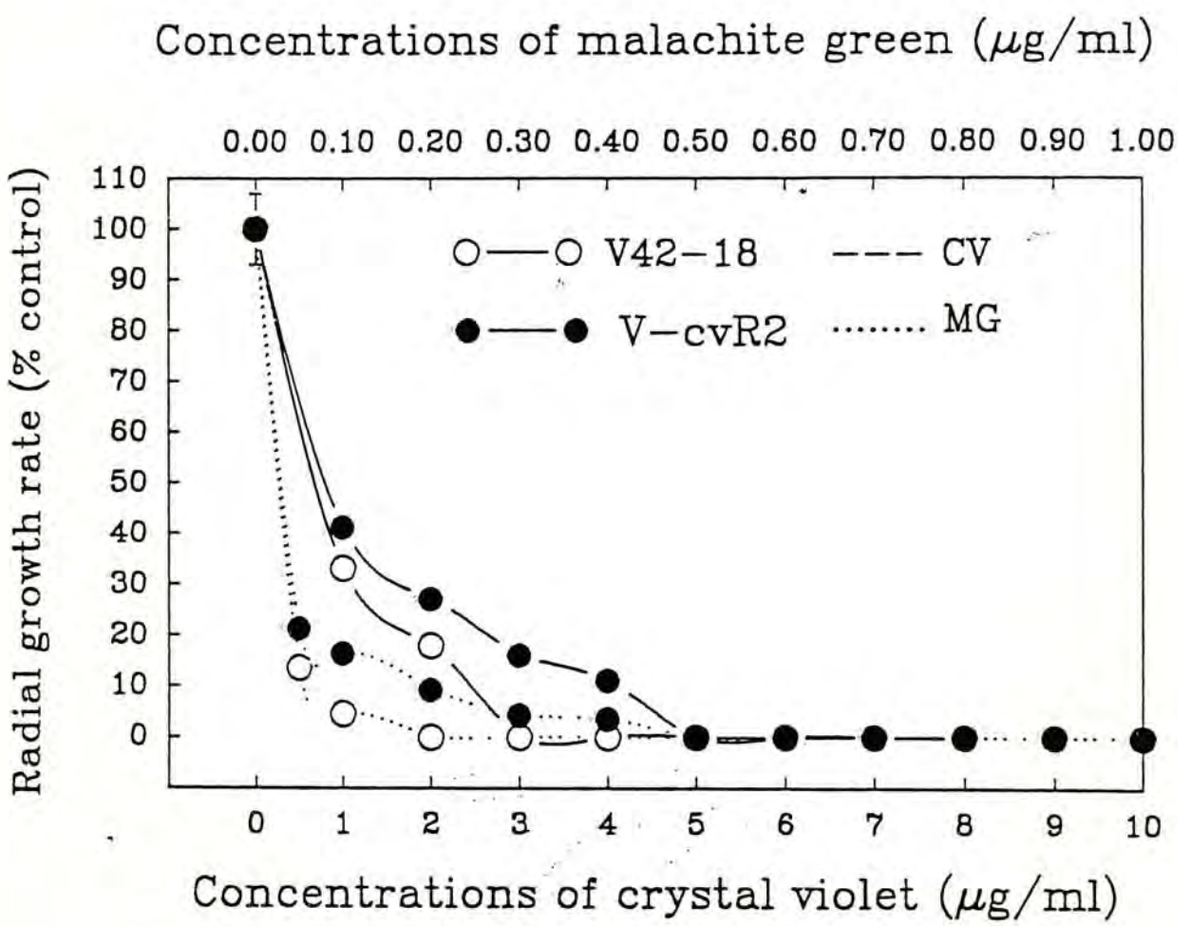


Fig. 4.16. Cross resistance of crystal violet resistant mutant *V-cvR2* and its wild type sensitive strain $V_{42\text{-}18}$ to malachite green supplemented to PDA.

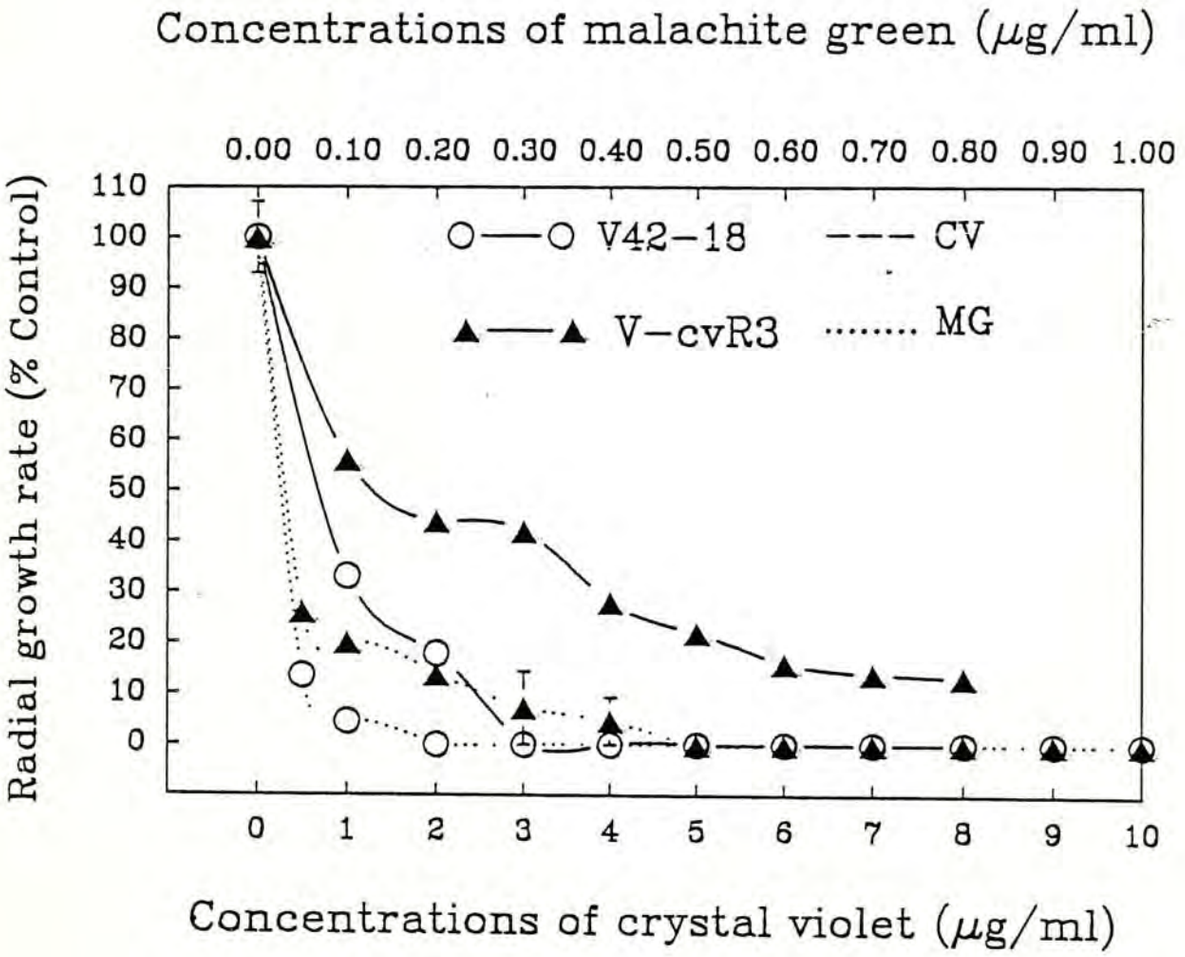


Fig. 4.17. Cross resistance of crystal violet resistant mutant *V-cvR3* and its wild type sensitive strain $V_{42\text{-}18}$ to malachite green supplemented to PDA.

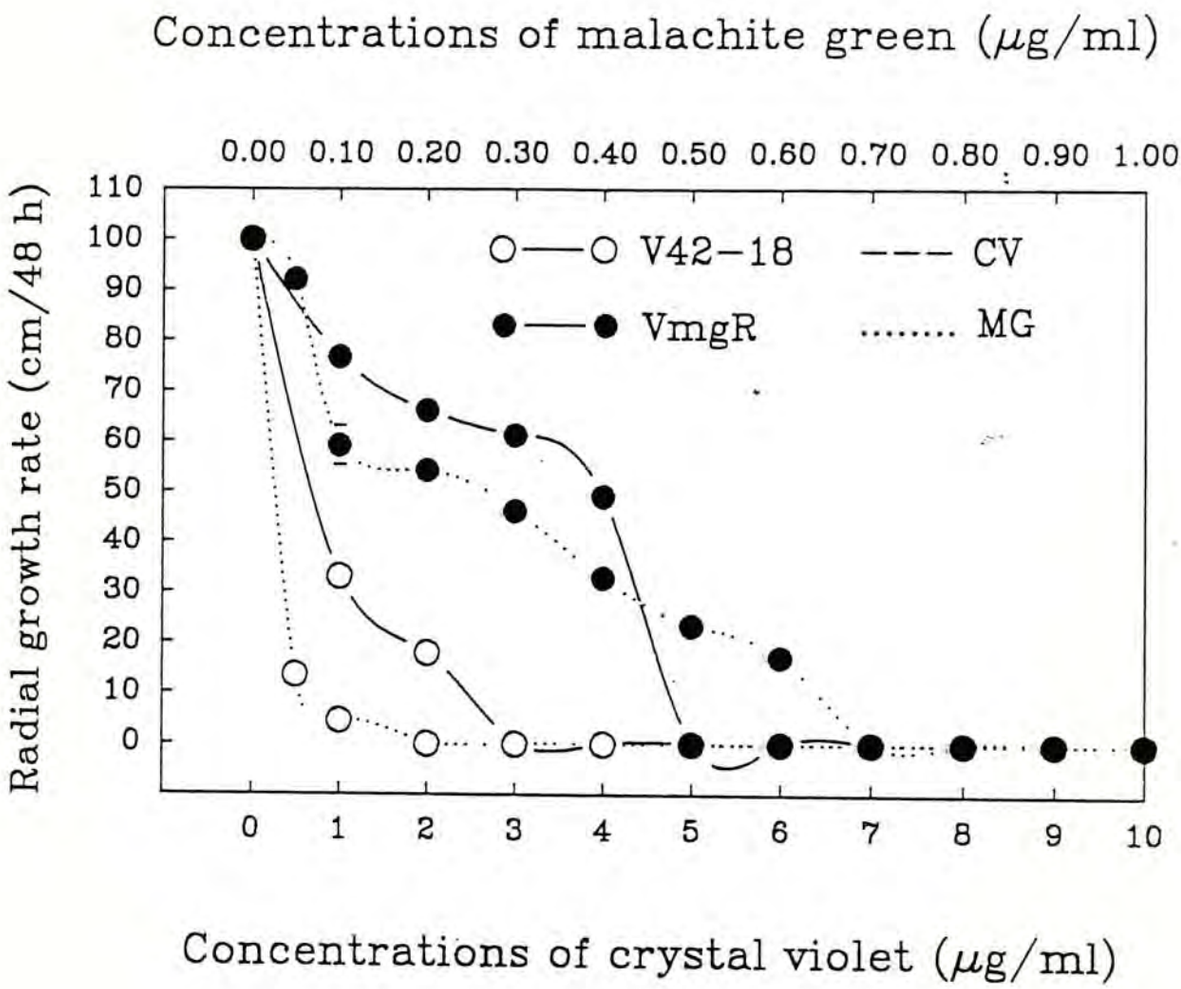


Fig. 4.18. Cross resistance of malachite green resistant mutant *V-mgR* and its wild type sensitive strain $V_{42\text{-}18}$ to crystal violet supplemented to PDA.

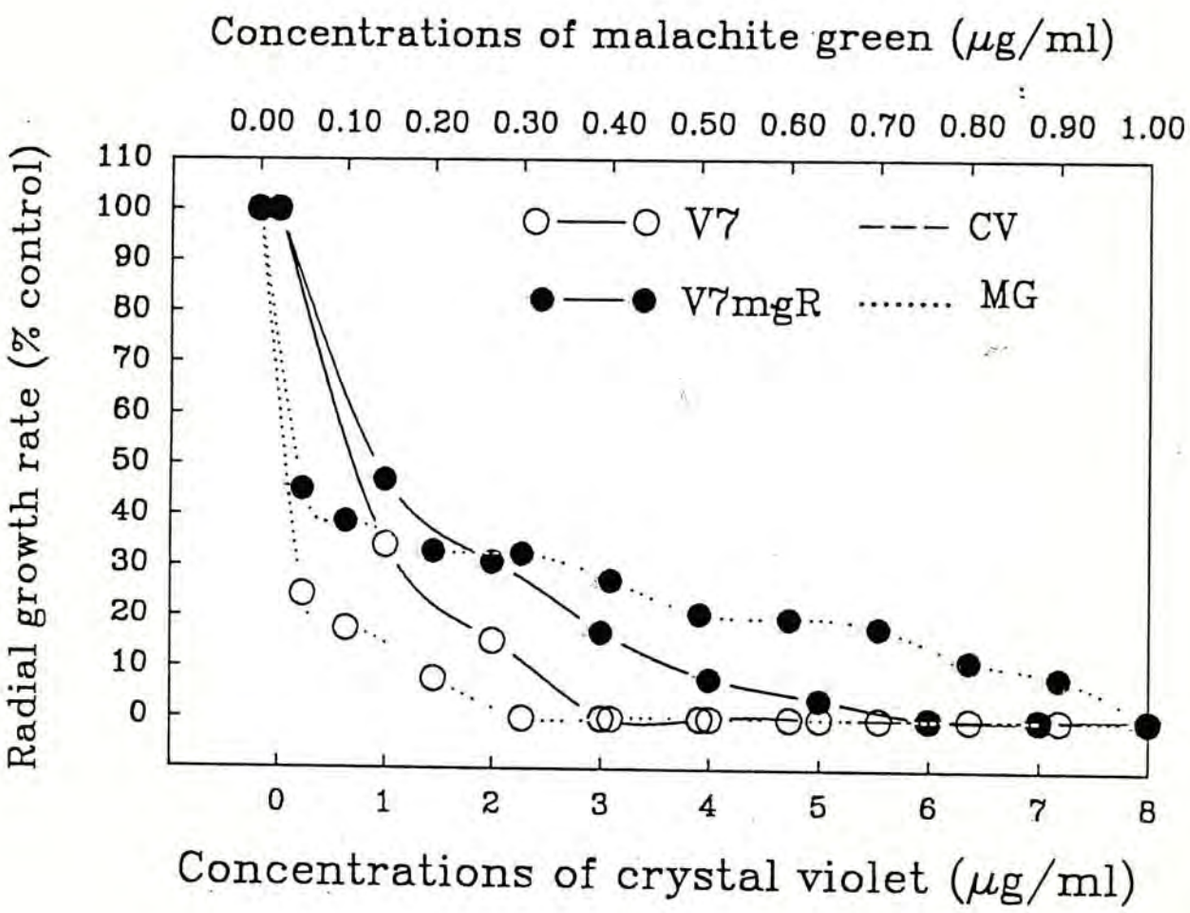


Fig. 4.19. Cross resistance of malachite green resistant mutant *V7-mgR* and its wild type sensitive strain V_7 to crystal violet supplemented to PDA.

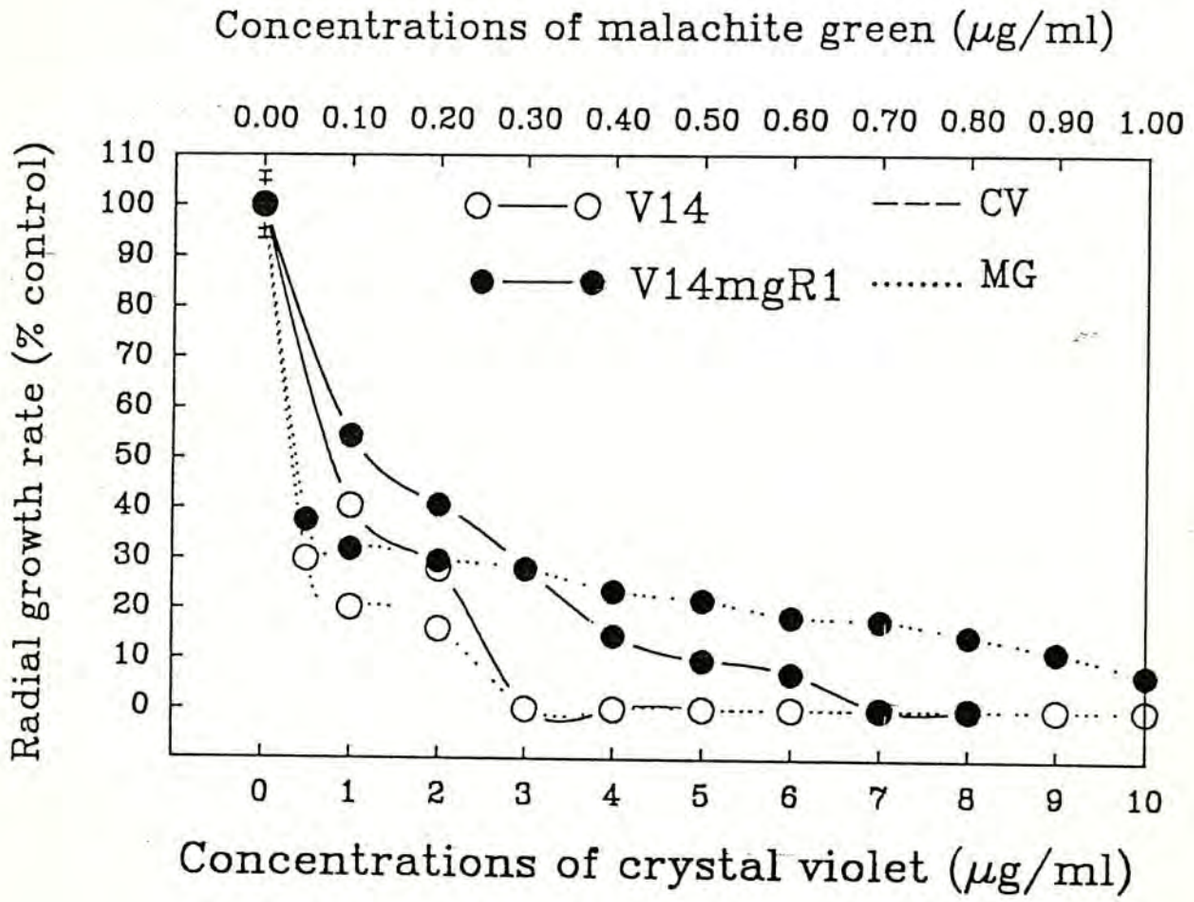


Fig. 4.20. Cross resistance of malachite green resistant mutant *V14-mgR1* and its wild type sensitive strain V_{14} to crystal violet supplemented to PDA.

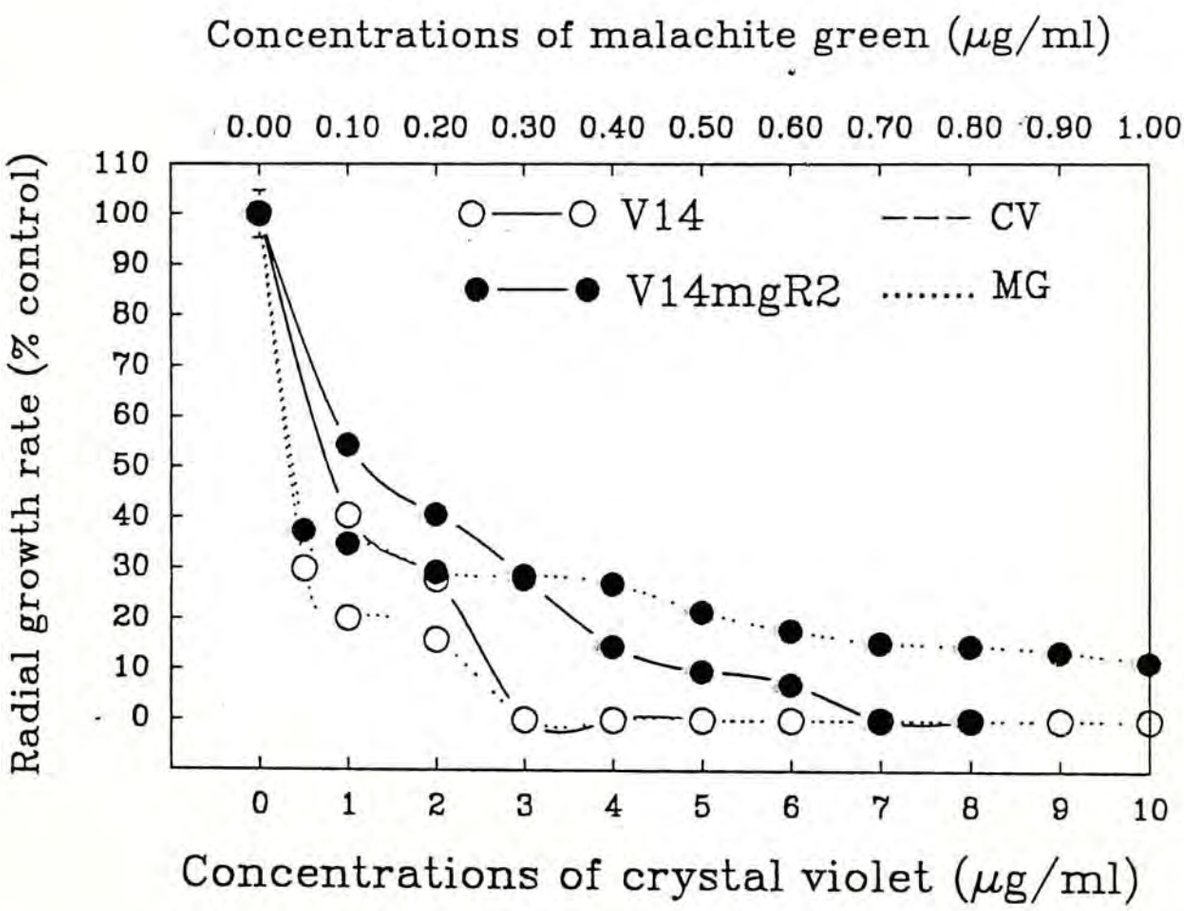


Fig. 4.21. Cross resistance of malachite green resistant mutant *V14-mgR2* and its wild type sensitive strain V_{14} to crystal violet supplemented to PDA.

(A)

(B)

Fig. 4.22. Fruiting bodies of (A) wild type strain $V_{42\text{-}18}$ and (B) crystal violet resistant mutant V-cvR1.

Results from fertility tests showed that all cycloheximide resistant mutants, either spontaneous or UV-induced, were sterile. Crystal violet and malachite green resistant mutants could produce sporophores, but most of them were aberrant. The characteristics of some resistant mutants obtained are summarized in Table 4.8. Although resistance could probably be due to an alteration in an uptake system, the results from osmotic sensitivity study showed that resistant mutants had the same response to sodium chloride as their wild type strains. They were unable to grow on PDA supplemented with 2% (W/V) sodium chloride.

Table 4.8. Characteristics of resistant mutants of *V. volvacea*.

Mutant	No.	MIC (μg/ml)		Fertility	Cross resistance properties at MIC		
		parent	mutant range	No. fertile /No. tested	cy	cv	mg
V-cyR	4	6	15-30	0/4	4	0	0
V7-cyR	2	10	30-40	0/2	2	0	0
V-cvR	3	3	5-10	3/3*	0	2	2
V-mgR	1	0.30	0.6	1/1	0	1	1
V7-mgR	1	0.25	1.0	1/1	0	1	1
V14-mgR	2	0.25	1-1.5	2/2	0	2	2

MIC: minimum inhibitory concentration
* aberrant sporophores
cy: cycloheximide
cv: crystal violet
mg: malachite green

For genetic analysis, "crosses" experiments were performed between the cycloheximide resistant mutants to see whether they could become fertile again. However, the results were negative (Table 4.9).

Table 4.9. Fertility test of "crossed" cultures between cycloheximide resistant mutants of *V. volvacea*.

Mutant	*V-cyR1*	*V-cyR2*	*V-cyR3*	*V7-cyR1*	*V7cyR2*
V-cyR1					
V-cyR2	-				
V-cyR3	-	-			
V-cyR4	-	-	-		
V7-cyR1	-	-	-	-	
V7-cyR2	-	-	-	-	-

"Cross" experiments were also performed between mutants with different markers. Meanwhile, presumptive "heterokaryotic hyphae" were taken from the contact zone between two different mutants on complete medium. Their resistances were further retested (Tables 4.10, 4.11 and 4.12). The method was used as described in Chapter 4.2.3.Two mm diameter agar plus of mycelia were placed at the centre of PDA plate containing different concentrations of tested compounds. In order to obtain accurate results as much as possible, the agar should be removed from the inocula. In this study, attempts have also been made to get progeny from the crossed cultures with different mutants. However, no fruiting bodies were obtained.

Table 4.10. Resistance test of "crossed" culture between mutants *V7-cyR1* and *V-mgR1*.

concentration (μg/ml)	*V7-cyR1*(a	*V-mgR1*(b	*V7-cyR1* x *V-mgR1*
PDA	++	++	++
CY10	+	-	+
CV3	-	+	+
MG0.2	-	+	+
CY10+CV3	-	-	+
CY12+CV3	-	-	+
CY14+CV3	-	-	+
CY16+CV3	-	-	+
CY18+CV3	-	-	+
CY20+CV3	-	-	+
CY30+CV3	-	-	-
CY40+CV3	-	-	-
CY10+CV4	-	-	+
CY10+CV5	-	-	-
CY10+CV6	-	-	-
CY10+MG0.2	-	-	+
CY10+MG0.3	-	-	+
CY10+MG0.4	-	-	+
CY10+MG0.5	-	-	+
CY10+MG0.6	-	-	-

CY: cycloheximide
CV: crystal violet
MG: malachite green
+: growth; ++: good growth; -: no growth
(a *V7-cyR1* was able to grow on PDA containing 40μg/ml CY, 2.5μg/ml CV and 0.15μg/ml MG, but failed to grow in higher concentrations of these inhibitors.
(b *V-mgR1* was able to grow on PDA containing 6μg/ml CY, 4μg/ml CV and 0.6μg/ml MG, but failed to grow in higher concentrations of these inhibitors.

Table 4.11. Resistance test of "crossed" culture between mutants *V7-cyR1* and *V-cvR1*.

Concentration (μg/ml)	*V7-cyR1*(a	*V-cvR1*(b	V7-cyR1 x *V-cvR1*
PDA	++	++	++
CY10	+	-	+
CV3	-	+	+
MG0.2	-	+	+
CY10+CV3	-	-	+
CY12+CV3	-	-	+
CY14+CV3	-	-	+
CY16+CV3	-	-	+
CY18+CV3	-	-	+
CY20+CV3	-	-	+
CY30+CV3	-	-	-
CY40+CV3	-	-	-
CY10+CV4	-	-	+
CY10+CV5	-	-	-
CY10+CV6	-	-	-
CY10+MG0.2	-	-	+
CY10+MG0.3	-	-	+
CY10+MG0.4	-	-	-
CY10+MG0.5	-	-	-
CY10+MG0.6	-	-	-

CY : cycloheximide
CV : crystal violet
MG: malachite green
+: growth; ++: good growth; -: no growth
(a *V7-cyR1* was able to grow on PDA containing 40μg/ml CY, 2.5μg/ml CV and 0.15μg/ml MG, but failed to grow in higher concentrations of these inhibitors.
(b *V-cvR1* was able to grow on PDA containing 6μg/ml CY, 5μg/ml CV and 0.4μg/ml MG, but failed to grow in higher concentrations of these inhibitors.

Table 4.12. Resistance test of "crossed" culture between mutants *V7-cyR1* and *V-cvR2*.

concentration (μg/ml)	*V7-cyR1*(a	*V-cvR2*(b	*V7-cyR1* x *V-cvR2*
PDA	++	++	++
CY10	+	-	+
CV3	-	+	+
MG0.2	-	+	+
CY10+CV3	-	+	+
CY12+CV3	-	-	+
CY14+CV3	-	-	+
CY16+CV3	-	-	+
CY18+CV3	-	-	+
CY20+CV3	-	-	+
CY30+CV3	-	-	+
CY40+CV3	-	-	-
CY10+CV4	-	-	+
CY10+CV5	-	-	+
CY10+CV6	-	-	-
CY10+MG0.2	-	-	+
CY10+MG0.3	-	-	+
CY10+MG0.4	-	-	+
CY10+MG0.5	-	-	-
CY10+MG0.6	-	-	-

CY : cycloheximide
CV : crystal violet
MG: malachite green
+: growth; ++: good growth; -: no growth
(a *V7-cyR1* was able to grow on PDA containing 40μg/ml CY, 2.5μg/ml CV and 0.15μg/ml MG, but failed to grow in higher concentrations of these inhibitors.
(b *V-cvR2* was able to grow on PDA containing 6μg/ml CY, 5μg/ml CV and 0.4μg/ml MG, but failed to grow in higher concentrations of these inhibitors.

4.3.5. Identification and characterization of auxotrophic mutants

By using UV mutagenesis and enrichment methods, several auxotrophic mutants were obtained in this investigation. No auxotrophic mutants grew on minimal medium. According to the methods of Holliday (1956), two auxotrophs derived from strain $V_{42\text{-}18}$ appeared to require aspartate (Table 4.13). This result was confirmed by the specific nutrient supplement added in minimal medium. Auxotrophs isolated from different strains were also identified by Holliday's method (Holliday, 1956).

Table 4.13. Characteristics of auxotrophic mutants of *V. volvacea*

Original strain	No. of auxotroph	Mutant codes	Nutrient requirement	Colony morphology	Fertility
$V_{42\text{-}18}$	2	*asp*$^-$	aspartate	N	F
		asp$^-$	aspartate	A	S

N: normal
A: abnormal
F: fertile
S: sterile

4.3.6. Analysis of genetic markers in progenies of mutants

In the present study, it is very difficult to analyze the genetic markers in mutant progenies since most of the mutants obtained are sterile or abnormal. Among them, *asp*$^-$1 and *VmgR, V14mgR1* mutant strains were chosen for study.

Table 4.14 illustrates the results of asp$^-$ strain progeny test. 31 out of 33 monosporous germlings retained their parental mutant phenotype. The two exceptional ones could grow on either minimal and complete media or minimal medium supplemented with aspartate.

Table 4.14. Progeny analysis of *asp*⁻ mutant strains*

Monosporous isolates tested	Marker			
	asp⁻		*asp*⁺	
	No.	%	No.	%
11	11	100	0	0
9	8	88.89	1	11.11
7	7	100	0	0
6	5	83.33	1	16.67
Total:33	31	93.94	2	6.67

* germination rate: 0.3 -0.5%.

Results of progeny analysis of *V-mgR* and *V14-mgR1* mutant strains are demonstrated in Table 4.15. Over 90% of progeny of mutants retained their parental marker phenotype.

Table 4.15. Progeny analysis of malachite green resistant mutants.

Mutant	No. of monosporous isolates tested	*MgR* marker			
		+		-	
		No.	%	No.	%
V-mgR	37	34	91.89	3	8.11
V14-mgR	24	23	95.83	1	4.17

+: with marker
-: without marker

4.4. Discussion

4.4.1. Significance of mutant induction in *V. volvacea*

Mutant induction has been reported in some edible fungi, such as *Agaricus bisporus* (Raper, *et al.*, 1972; Challen and Elliott, 1987; Challen *et al.*, 1989a; 1989b), *Agrocybe aegerita* (Labarere *et al.*, 1989), *Pleurotus* (Leallara, 1977; Imbernon and Labarere, 1989), *Volvariella bombycina* (Chiu and Chang, 1987). At present, information on mutant induction of *V. volvacea* is limited. Barroso *et al.* (1988) failed to induce stable chloramphenicol and tetracycline resistant mutants of *V. volvacea* by UV mutagenesis. In addition, Mukherjee and Sengupta (1986) also found that most of the putative mutants quickly reverted to the wild type on subsequent subculture. They also reported that no morphological or auxotrophic mutants could be obtained from protoplasts by chemical mutagenesis. It is very difficult to obtain stable mutants of *V. volvacea* probably due to multinucleate nature of hyphal cell and thick-walled basidiospores.

In spite of these difficulties, attempts were still made to induce mutations since mutant markers are of paramount importance and useful in genetic analysis. By using mutant markers, the sexuality pattern and the mechanism of *V. volvacea* could be investigated and analyzed.

If reliable mutants were obtained, several unanswered problems could be analyzed. For example, if *V. volvacea* is indeed a primary homothallic species, i.e. self-fertile without crossing, the mutant markers should not be lost in their progenies from basidiospores of fruiting bodies unless other mutations occurred. However, if the mutant markers are segregated in the progeny, the genetic make-up of basidiospores should be analyzed by the ratio of marker segregation. Without mutant markers, such analysis becomes impossible.

Furthermore, without mutant markers, it is impossible to distinguish between heterokaryotic and homokaryotic hyphae, because no clamp connection has been found in *V. volvacea*. If two different mutants are allowed to mate with each other by nutritional forcing or other methods, heterokaryotic strains will be obtained and studied.

4.4.2. Methods for mutant induction

The effects of UV irradiation on mycelial fragments and basidiospores were investigated. It was found that the mycelia were more susceptible to UV-irradiation than the spores. For the strain $V_{42\text{-}18}$, a UV dose causing 99% mortality of mycelial fragment was about 80 seconds but in spores, it was about 200 seconds. The possible reason is that cell wall of spores is usually thicker and more complicated than that of mycelia, this could make it more difficult for UV to penetrate to the spore nuclei. Challen and Elliott (1987) have demonstrated that hyphal fragments were used for mutagenesis in preference

to spores. The use of hyphal fragments should favour the retention of parental characteristics and fragment regeneration is much more uniform than spore germination. Nevertheless, the hyphal cell is multinucleate and masks mutation by the presence of wild type nuclei. The occurrence of hyphal anastomosis frequently can enhance the distribution of wild-type nuclei and their masking effects (Volk and Leonard, 1989). Moreover, hyphal fragments are not uniform in size, and this coupled with the multinucleate nature of the cells would hinder the obtaining of mutations. Although basidiospores of this organism are thick-walled, most of them were uninucleate (Chang, 1969). Therefore, both hyphal fragments and basidiospores were used to induce mutants in the present study.

For chemical mutagenesis, two mutagens, acriflavine and ethidium bromide were used. Acriflavine belongs to the frame shift mutagen. It can mainly stimulate unequal crossing-over by intercalation of its molecule between adjacent DNA bases, thus forcing them further apart than normal. Another genetic effect of acriflavine is its ability to give rise to cytoplasmic variants (Fishbein *et al.*, 1970; Gardner and Snustad, 1984). Ethidium bromide is also a powerful mutagen, it can promote the loss of DNA in the cell, especially mitochondrial DNA.

In order to obtain auxotrophs efficiently, mutant enrichment using ethidium bromide was performed after UV treatment. This technique takes the advantage of the fact that ethidium bromide promotes the loss of mitochondrial DNA of growing cells much more rapidly than non-growing cells (Fincham and

Day, 1971; McCusker and Haber, 1988). In this study, basidiospores were first irradiated by UV and inoculated in liquid minimal medium. Only prototrophic spores could germinate and grow in minimal medium and then ethidium bromide was added to kill the germlings. After these treatments, all survivors which were potential auxotrophs were further tested with complete and minimal agar medium. By using this enrichment method, three auxotrophic mutants have been obtained and the mutation frequency was 2.1×10^{-6}.

4.4.3. Resistant mutants of *V. volvacea*

In this study, cycloheximide, crystal violet, malachite green, potassium chlorate and sodium chloride were used to screen for resistant mutants.

Cycloheximide is an inhibitor of protein synthesis in a variety of eukaryotic system. Mutations altering the cycloheximide sensitivity of ribosomes have been reported in some fungi: *Saccharomyces cerevisiae* (Cooper *et al.*, 1967; McCusker and Haber, 1988), *Schizosaccharomyces pombe* (Ibrahim and Coddington, 1976; Shneyour *et al.*, 1978), *Podospora anserina* (Crouzet *et al.*, 1978), *Neurospora crassa* (Neuhäuser *et al.*, 1970; Vomvoyanni, 1974) and *Saccharomyces fragilis* (Siegel and Sisler, 1965). In *Coprinus cinereus*, a large number of cycloheximide resistant mutants has been isolated after UV treatment of oidia. These mutants belong to the *cy-2* locus which maps on linkage group II (North, 1982). *In vitro* studies have shown that the *cy-2*^{r} mutation affects the

cytoplasmic ribosomes (Traynor *et al.*, 1986), specifically the 60S subunit (Sardharwalla and North, 1986). Recently, North (1989) reported that cytoplasmic ribosomes from cycloheximide sensitive and resistant strains of *Coprinus cinereus* were dissociated with ethylene diamine tetracetic acid (EDTA) and analyzed by sucrose density-gradient centrifugation. It was found that mutant 60S subunit was reduced in amount in proportion to the 40S subunit and sedimentation was less rapid than the wild type.

With reference to Table 4.6, 89 putative cycloheximide resistant mutants were obtained after UV irradiation. Among them, only 7 had a stable mutant phenotype. It is worth noticing that all cycloheximide resistant mutants, including spontaneous and UV induced ones obtained in this study were sterile. They could not produce any fruiting body in cotton waste compost bags. These results suggested that there might be some relationships between the 60S ribosome and the sterility since cycloheximide resistant mutations can affect the cytoplasmic ribosomes, especially the 60S subunit.

In the present study, crystal violet and malachite green were also used to screen resistant mutants. Crystal violet (N,N,N',N',N",N"-hexamethylparaosaniline) belongs to the triphenylmethane dyes, which have been extensively use in human and veterinary medicine, as a biological stain, and as a textile dye. Crystal violet has been shown to be carcinogenic and responsible for promotion of tumor growth in some species of fish (Au *et al.*, 1978; 1979). It was reported that the wood-rotting fungus *Phanerochaete*

chrysosporium can degrade crystal violet with its lignin-degrading system (Bumpus and Brock, 1988). In part, the lignin-degrading system consists of a number of peroxidases that are secreted by the fungus under the conditions of nutrient limitation. These peroxidases are commonly referred to as ligninases or lignin peroxidases and have the ability to catalyze the depolymerization of lignin as well as the initial oxidation of a wide variety of other compounds. The first reactions in the oxidative biodegradation of crystal violet are N-demethylation reactions catalyzed by a lignin peroxidase. It was also reported that oxidative red yeasts can degrade crystal violet (Kwasniewska, 1985). However, *V. volvacea* was sensitive to crystal violet. None of the strains tested could grow on 3 μg/ml crystal violet supplemented to PDA (Fig.4.6.). It was suggested that the sensitivity to crystal violet was probably due to the lack of a lignin degrading system in *V. volvacea.*

Malachite green also belongs to the triphenylmethylparao-saniline dyes. The cross resistance between the *cvR* and *mgR* mutants can probably be attributed to their chemical similarity.

In order to get double markers, potassium chlorate was used to screen resistant mutants. The principle is that although chlorate itself is not toxic, it is converted by sensitive strains to chlorite, which is toxic (Correll *et al.*, 1987). The enzyme which facilitates this conversion from chlorate to chlorite is nitrate reductase, which under normal conditions acts in the conversion of nitrate to nitrite. The basis of resistance is that a resistant strain cannot convert chlorate

to chlorite and is also not able to convert nitrate to nitrite; hence they cannot grow on media with nitrate as the sole nitrogen source (Volk and Leonard, 1989). In this study, the effect of potassium chlorate on mycelial growth of ten strains and germination rate of basidiospores have been investigated (Fig.4.11 and 4.12). All strains tested were unable to grow on PDA containing 5% potassium chlorate. However, stable resistant isolates were never recovered from UV-irradiated screening, as was the case with the sodium chloride resistant mutants.

4.4.4. Auxotrophic mutants of *V. volvacea*

As in the case of *Agaricus bisporus* (Challen and Elliott, 1987) and *Morchellae sculenta* (Volk and Leonard, 1989), induction and selection of auxotrophs is both difficult and time consuming mainly due to the multinucleate nature of hyphal cells. In the present study, over 5,500 isolates from mycelial fragments after UV or chemical treatments have been tested. However, no stable auxotrophic mutants have been obtained.

Although the germination rate of basidiospores in *V. volvacea* was poor and erratic, they were still used to induce mutants since most of them were uninucleate. By UV mutagenesis and mutant enrichment using ethidium bromide, only one fertile auxotroph has been obtained (Table 4.13). Generally, auxotrophic mutants differed in growth from their parental strain. They often have abnormal morphology and grow poorly on CM medium.

4.4.5. Study on the sexuality pattern of *V. volvacea* by analyzing mutant progeny

Although *V. volvacea* has been cultivated for many years, the sexual characteristics have remained obscure. There are two different explanations given: primary homothallism or secondary homothallism. Because clamp connections are absent in *V. volvacea*, genetic markers are very important for proving the self-fertility of monosporous isolates.

Auxotrophic mutations are usually recessive to their corresponding wild alleles and are therefore expressed only in the haploid or homoallelic condition in which case the mutant strains are incapable of growing on minimal medium and can only grow when provided with their specific requirement.

Because mutants induced by UV usually have reduced growth rate and sporulate poorly, it is difficult to collect spores from the mutant strain *asp*$^-$. Only 33 monosporous isolates from 3 fruiting bodies were analyzed. The results from Table 4.14 demonstrate that all the mutant progenies retained the mutant phenotype except two morphologically abnormal isolates. Moreover, in the case of mutant strains *V-mgR* and *V14-mgR*, over 90% of its monosporous isolates were also resistant to malachite green. In addition, 10% of the mutant progenies did not have mutant markers. This may have been due to the formation of

aneuploids which might result in the loss of the DNA segment or chromosome(s) carrying the marker. Meanwhile, the possibility of the occurrence of back mutation cannot be excluded. Based on these results of mutant progeny analysis, it could be concluded that *V. volvacea* is a primary homothallic species.

Although clamp connections are absent in this edible fungus, heterokaryosis was found since the crossed cultures after anastomoses had two resistances derived from their parental mutant strains (Tables, 4.10; 4.11 and 4.12). It seems very simple but heterokaryosis has never been confirmed in *V. volvacea* by using mutant markers. It indicates the importance of mutant markers and the difficulty of mutant induction. This result would also be supported by the study of single and joint segregation of biochemical loci in intraspecific crossing of *V. volvacea* (Royse, *et al.*,1987). According to the information of heterokaryosis, a practical selective breeding program for *V. volvacea* is available. Firstly, single spore isolates derived from the strains with desirable commercial traits would be obtained. Then dual cultures are made to produce new hybrids from which it is possible to select and obtain ideal strains. Of course, there is also considerable potential for the application of protoplast fusion technique and DNA transformation for strain improvement.

Since no fruiting bodies were obtained from the crossed cultures with different mutants in this study, segregation of different markers in their progenies cannot be analyzed. It thus remains to explain the mechanism of

variation found in monosporous isolates. Further studies are needed to elucidate whether the phenotypic variation is due to genetical variation or physiological changes.

Recently, a new method, Arbitrarily Primed Polymerase Chain Reaction (AP-PCR) was developed (Welsh and McClelland, 1990; Welsh, *et al.*, 1991; Williams, 1989). This new DNA polymorphism analysis is based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence. DNA polymorphisms amplified by AP-PCR are useful as DNA fingerprinting genetical markers. Williams *et al.* (1990) proposed to call these markers RAPD markers (pronounced 'rapid', for Random Amplified Polymorphic DNA). AP-PCR offers several advantage over classical mutant induction. It can amplify a small amount of DNA, such as from a single human sperm, a single human hair and a single spore of *Neurospora tetrasperma,* even from the spores which could not be germinated. Moreover, AP-PCR method uses primers chosen without regard to the sequence of the genome to be fringerprinted. Each primer gives different patterns of AP-PCR products. Therefore, the sexual pattern of *V. volvacea* would be studied by analyzing the DNA polymorphisms of monosporous isolates derived from the same sporocarp. If *V. volvacea* is really a primary homothallic species, and if the fruiting body is produced from a self-fertile isolate, the DNA pattern generated by AP-PCR would be the same unless other mutations occur. As a whole, this method provides a possible means to identify whether the sporocarp is actually produced from a monosporous isolate. It can also detect the occurrence of mutations by analyzing the change in DNA polymorphic markers.

4.5. Summary

1. In order to obtain genetic markers, basidiospores and mycelial fragments were treated by UV or chemical mutagens. Auxotroph enrichment using ethidium bromide was also used.

2. Seven resistant mutants and two auxotrophic mutants were obtained.

3. Instability and sterility of the mutants are the main problems in the present study. It is mainly due to the multinucleate nature of cells and multiple sited mutation of mutagen.

4. Results from progeny analysis of mutant strains, *asp*$^-$, *V-mgR* and *V14-mgR*, showed that over 90% of the progenies retained their parental mutant phenotype. It could be concluded that *V. volvacea* is a primary homothallic species. In addition, 10% of the mutant progenies did not have mutant markers. This may have been due to the formation of aneuploids which might result in the loss of the DNA segment or chromosome(s) carrying the marker. Meanwhile, the possibility of the occurrence of back mutation cannot be excluded.

5. The occurrence of "Heterokaryosis" in *V. volvacea* was found since the "crossed" cultures had two resistances derived from their parental mutant strains.

Chapter five: Cytological studies on variations of *Volvariella volvacea*

5.1. Introduction

Although a few cytological studies have been devoted to investigate the sexuality and life cycle of *V. volvacea* (Chang and Ling, 1970; Chang and Yau, 1971; Li, 1977), it is difficult to explain the possible source of variation by pure description of cell morphology. At the same time, artifacts, commonly occur when classical staining techniques are employed, often giving rise to different arguments or interpretations of the results.

The proposition of heteroploidy as a mechanism of variability among fungi (Tolmsoff, 1983) has attracted people's attention. Evidences for heteroploidy were reported in some fungi, such as *Neurospora crassa* (Petes, 1980), *Aspergillus nidulans* (Käfer and Upshall, 1973; Gabrielli and Azevedo, 1980), *Saccharomyces cerevisiae* (Klapholz and Esposito, 1980; Petes, 1980; Campbell *et al.*, 1981), *Armillaria mellea* (Peabody *et al.*, 1978; Peabody and Peabody, 1984; Anderson *et al.*, 1985) and *Phytophthora infestans* (Therrien and Ritch, 1989). *Coprinus cinereus* (Murakami, 1989). Cytological consideration is also important in exploring the mechanism of variation in *V. volvacea*.

One of the goals of the present cytological investigation was to establish

a better staining method for observing the nuclear behaviour in the small nuclear size of *V. volvacea* cells, especially the thick-walled basidiospores. Furthermore, microscopic autoradiography was employed to confirm the results obtained by general staining method. Comparative studies on cytological differences of the self-fertile, self-sterile isolates and their parental strain $V_{42\text{-}18}$ were performed. The spore pattern on basidium was also examined by means of scanning electron microscope. To address the question whether any aneuploids were formed by atypical cell division in *V. volvacea*, the ploidy of hyphal cells and basidiospores was studied by measuring the DNA content with microscopic photometer.

5.2. Materials and methods

5.2.1. Strains

V. volvacea strains $V_{42\text{-}18}$ and V_7, originated from Hong Kong and Malaysia respectively, were examined. The culture medium used was as described in Chapter three. Cultures were incubated at 32°C for growth and stored at 15 °C. Both hyphal cells and basidiospores were studied.

5.2.2. Feulgen staining method

All specimens were stained by the modified Feulgen procedure of

Peabody's method (1984). 1N HCl, 5N HCl and 45% glacial acetic acid have been used to observe the hydrolysis results and the optimum hydrolysis time was determined by examining the results from different hydrolysis times, ranging from 1 to 8 minutes at 60°C.

5.2.3. Fluorescent staining of nuclei with DAPI

4',6-diamidino-2-phenylindole (DAPI), being a nucleic acid-specific dye, was used for fluorescent staining of nuclei. Specimens were prepared in a manner as described previously (Williamson and Fennell, 1975; TeBeest *et al.*, 1989). A Nikon Biophot (Nikon, Tokyo, Japan) epifluorescent microscope equipped with 100-W mercury vapour lamp was utilized. Observations were made by using filter combination UV-1A, giving a peak of excitation light between 330 and 380 nm.

5.2.4. Microscopic autoradiography

Microautoradiography was prepared with a modified method described by Li and Liu (1987) and Liu and Li (1987). 2 μ Ci / ml ^{3}H-thymidine (Sigma), being DNA precursor, were used to label nucleus. Nuclear emulsion used was N-4 (Beking Chemical works). "Slide Exposure" was undertaken in dark at 4°C for 12 days. For development and fixation, Kodak D19 and F5 were chosen respectively.

5.2.5. Scanning electron microscopic examination

To observe the spore pattern of basidia, small pieces of gills (2 to 4 mm^2) from fresh normal sporophore of strains V_{34}, V_{35} and $V_{42\text{-}18}$ were fixed with 3% glutaraldehyde and post-fixed in 2% osmium tetroxide. Both fixatives were dissolved in 0.2 M sodium cacodylate buffer (pH 7.2) or 0.2 M phosphate buffer (pH 7.2). After fixation, all specimens were dehydrated via an up-graded alcohol series to absolute ethanol. They were then transferred into liquid carbon dioxide for critical point drying. The specimens were sputter-coated with gold and observed with a JOEL model JSM-35 scanning electron microscope (JOEL, TOKYO, JAPAN).

5.2.6. DNA measurement

For measuring the content of nuclear DNA, all slides were prepared by DAPI-DNA staining method as mentioned above. DNA, expressed in arbitrary units (a.u.), was determined for individual DAPI-stained nuclei with a Nikon Biophot epifluorescent microscope fitted with a P1 microphotometer. The procedures were similar to that performed by Peabody and Peabody (1984).

It was important to know the rate of fluorophore photobleaching *in situ* so that all quantitative measurements could be made within the time period of maximum emission. Based on the results of Staples *et al.* (1984), fading under experimental conditions was consistent, with an exponential photobleaching

decay time greater than 1500 S. In this study, all nuclear fluorescence data were recorded in less than 1 S. All the data obtained were analyzed by using the statistical package (SPPSS/PC+) at the computer centre of the Chinese University of Hong Kong.

5.3. Results

5.3.1. Number of nuclei in *V. volvacea*

Vegetative cells of *V. volvacea* are multinucleate (Fig.5.1). The nuclei seem to be randomly distributed within the cells. The results of observing DAPI-stained nuclei in vegetative hypha are illustrated in Table 5.1. The number of nuclei per cell varies widely in the range from two to more than forty. The most frequent number was between fifteen to twenty (Figs.5.2 and 5.3).

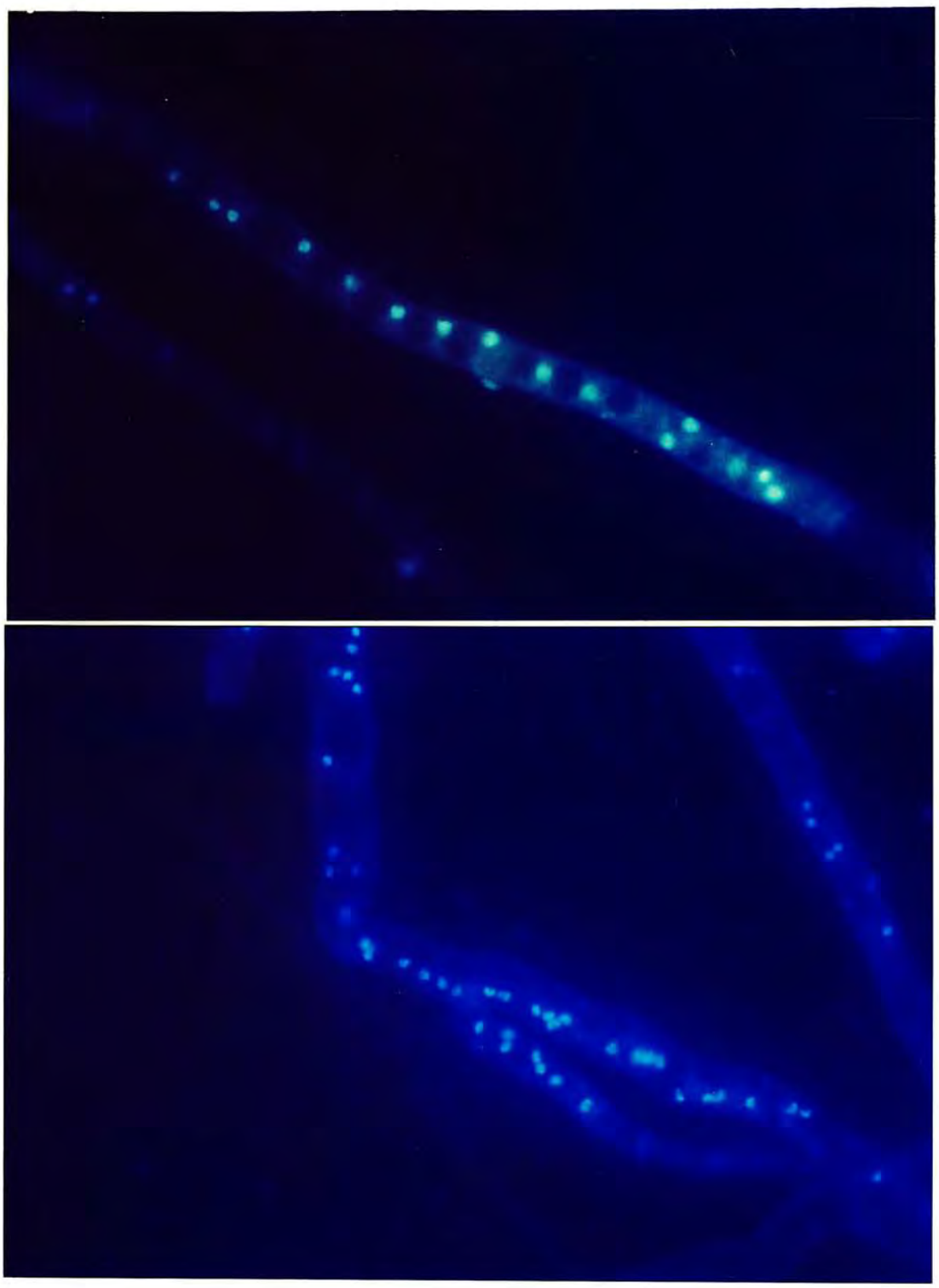


Fig. 5.1. Vegetative hyphae of *V. volvacea* showing they are multinucleate.

Table 5.1. Number of nuclei in vegetative hypha of *V. volvacea*

Strains	No. of cells observed	Range of number	Mean ± S.E.M.
$V_{42\text{-}18}$	47	2 - 48	21.98 ± 1.65
V_7	45	2 - 52	20.73 ± 1.91

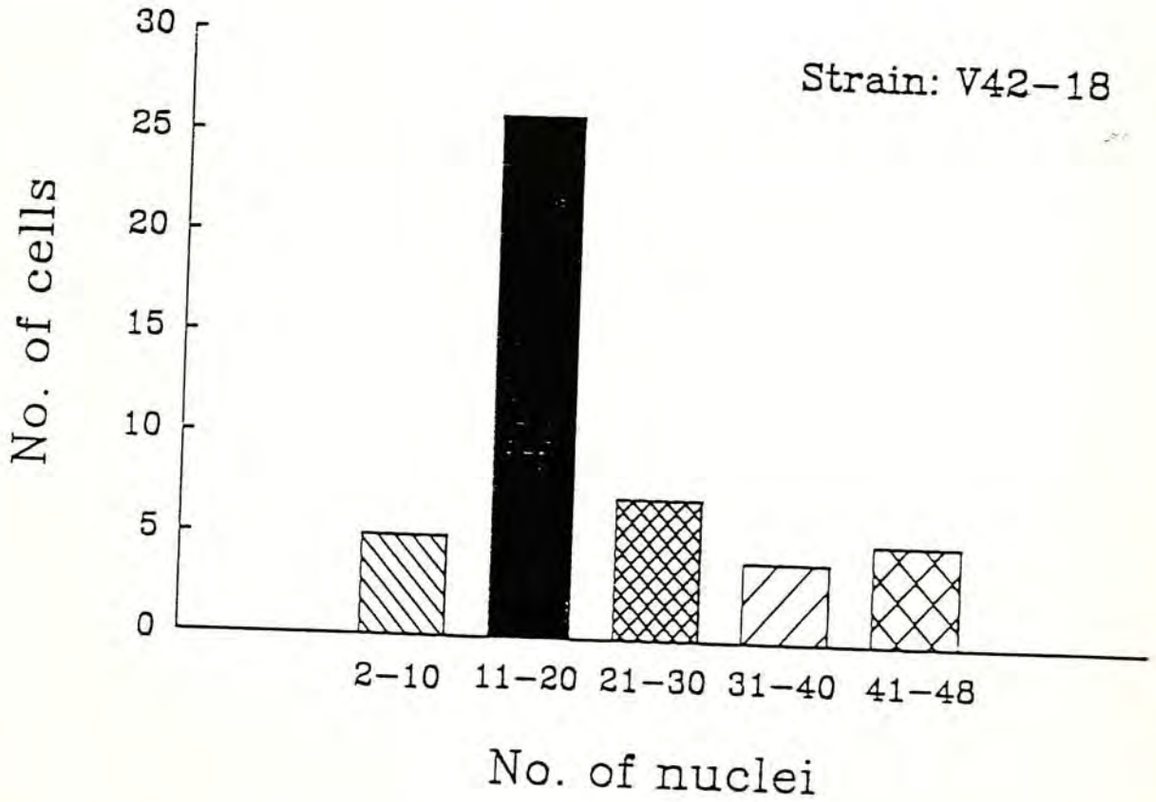


Fig. 5.2. Distribution of hyphal cells with different number of nuclei (strain: V42-18).

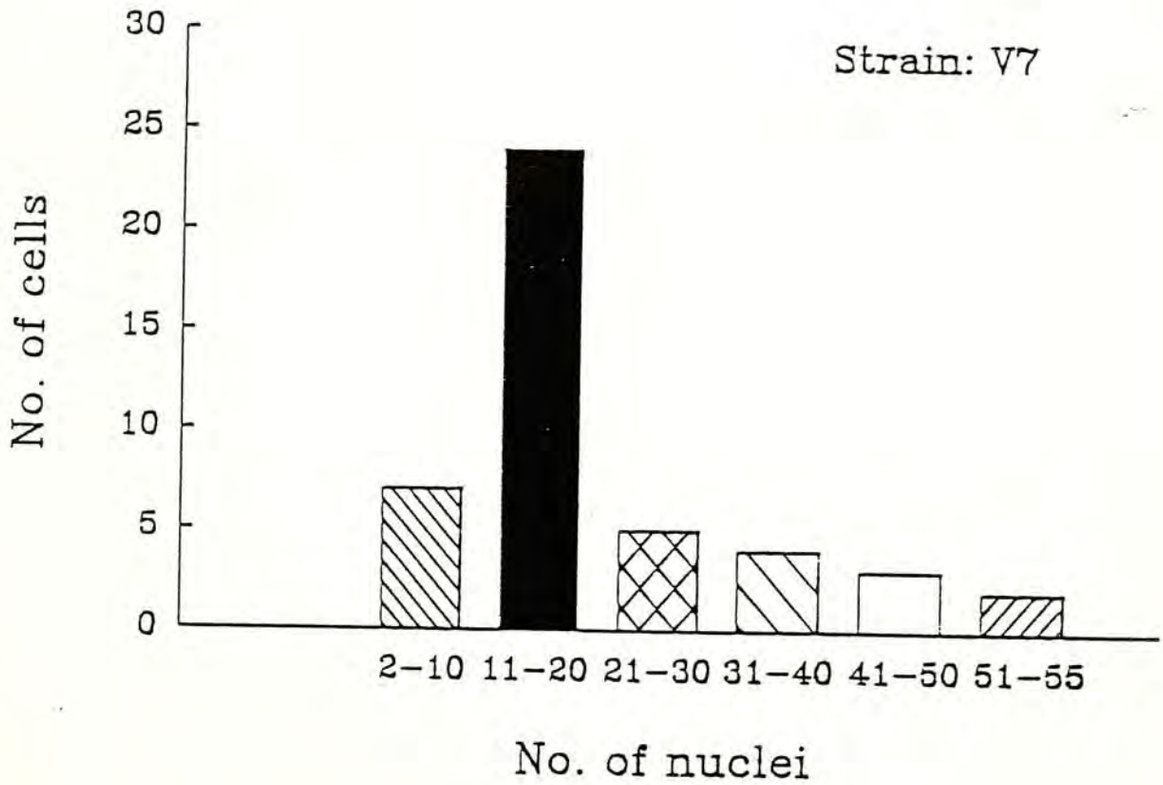


Fig. 5.3. Distribution of hyphal cells with different number of nuclei (strain: V_7).

The number of DAPI-stained nuclei in basidiospores was also observed (Fig. 5.4). In the strain $V_{42\text{-}18}$, 84.91% of the spores were uninucleate; 8.9% were binucleate; 4.07% spores contained 3 nuclei and 0.43% spores had four nuclei. In addition, 1.68% spores were enucleate, no nucleus was found under the microscope.

Table 5.2. Number of nuclei in basidiospores of *V. volvacea*

Strains	No. of fruit bodies observed	No. of spores observed	No.of nuclei per spore (Mean% ± S.E.M.)				
			0	1	2	3	4
$V_{42\text{-}18}$	3	935	1.68 ±0.29	84.91 ±1.54	8.90 ±0.70	4.07 ±0.74	0.43 ±0.11
V_7	3	648	1.39 ±0.71	86.51 ±0.71	10.57 ±0.89	1.28 ±0.15	0.26 ±0.04

Basidiospores of *V. volvacea* are small, the average in length is 7μm to 9μm while the wide end is about 5μm (Chang, 1965b). Because of the small size of nucleus, autoradiography was used to confirm whether the DAPI-stained granules were really nuclei. Totally 31 spores were observed. It was found that 26 spores (84%) had one nucleus. The results were in agreement with those of DAPI-staining method. Fig.5.5shows the microautoradiograph of basidiospores, nucleus is clearly observed in the basidiospore.

Fig. 5.4. Uninucleate and binucleate basidiospores of *V. volvacea*.

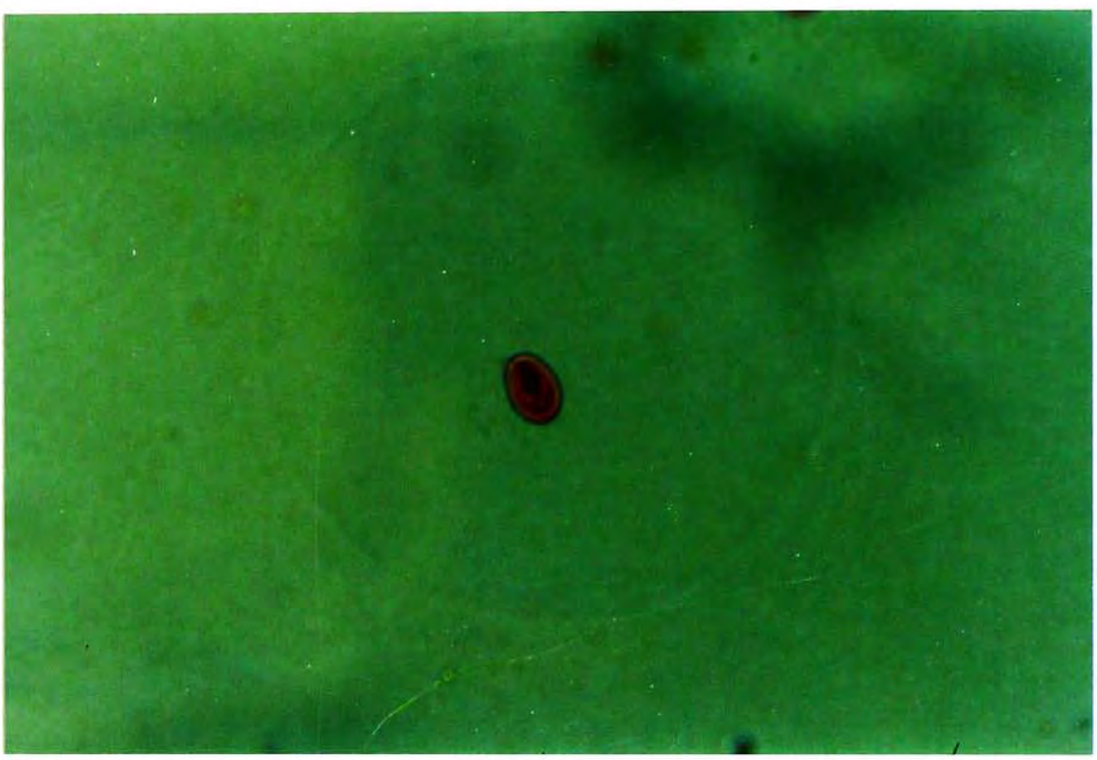


Fig. 5.5. Microautoradiograph of basidiospore of *V. volvacea* showing the nucleus labelled by ^{3}H-thymidine.

5.3.2. Comparative study on cytological differences between self-fertile and self-sterile monosporous isolates

Wild type strain $V_{42\text{-}18}$ and its monosporous isolates F-1 and SN-1, SA-1 were studied. Table 5.3 demonstrates the main cytological differences between self-fertile and self-sterile monosporous isolates. It was found that there were no significant differences between self-fertile (F-1) and self-sterile isolate (SN-1) with normal colony morphology. However, abnormal sterile isolate (SA-1) had few hyphal branches with compact morphology.

Table 5.3. Cytological differences of the self-fertile, self-sterile isolates and their parental strain V_{42-18}.

Monosporous isolate	V_{42-18} F1 (self-fertile)		SN-1 (self sterile)	SA-1 (self sterile)
General pattern of mycelia	in a network form			parallel to each other
Density of mycelia	scattered			compact
Hyphal branches	many			few
No. of nuclei per cell				
Range	2-48	2-50	2-48	1-10
Mean	21	22	16	5

F-1 : self-fertile isolate derived from strain V_{42-18}
SN-1: self-sterile isolate with normal colony morphology derived from strain V_{42-18}
SA-1: self-sterile isolate with abnormal colony morphology derived from strain V_{42-18}

5.3.3. Spore patterns on basidia

In the strain $V_{42\text{-}18}$, 239 mature basidia were examined. Most of them were tetrasporic or with 4 sterigmata (Fig. 5.6A). However, different spore patterns on basidia were also found. They were bisporic (Fig. 5.6B), trisporic (Fig. 5.6C) and pentasporic (Fig. 5.6D). Occasionally, some spores were fused together (Figs. 5.6E and 5.6F). These phenomena also occurred in the strains V_{34} and V_{35}. Distribution of different spore patterns on basidia are shown in Table 5.4.

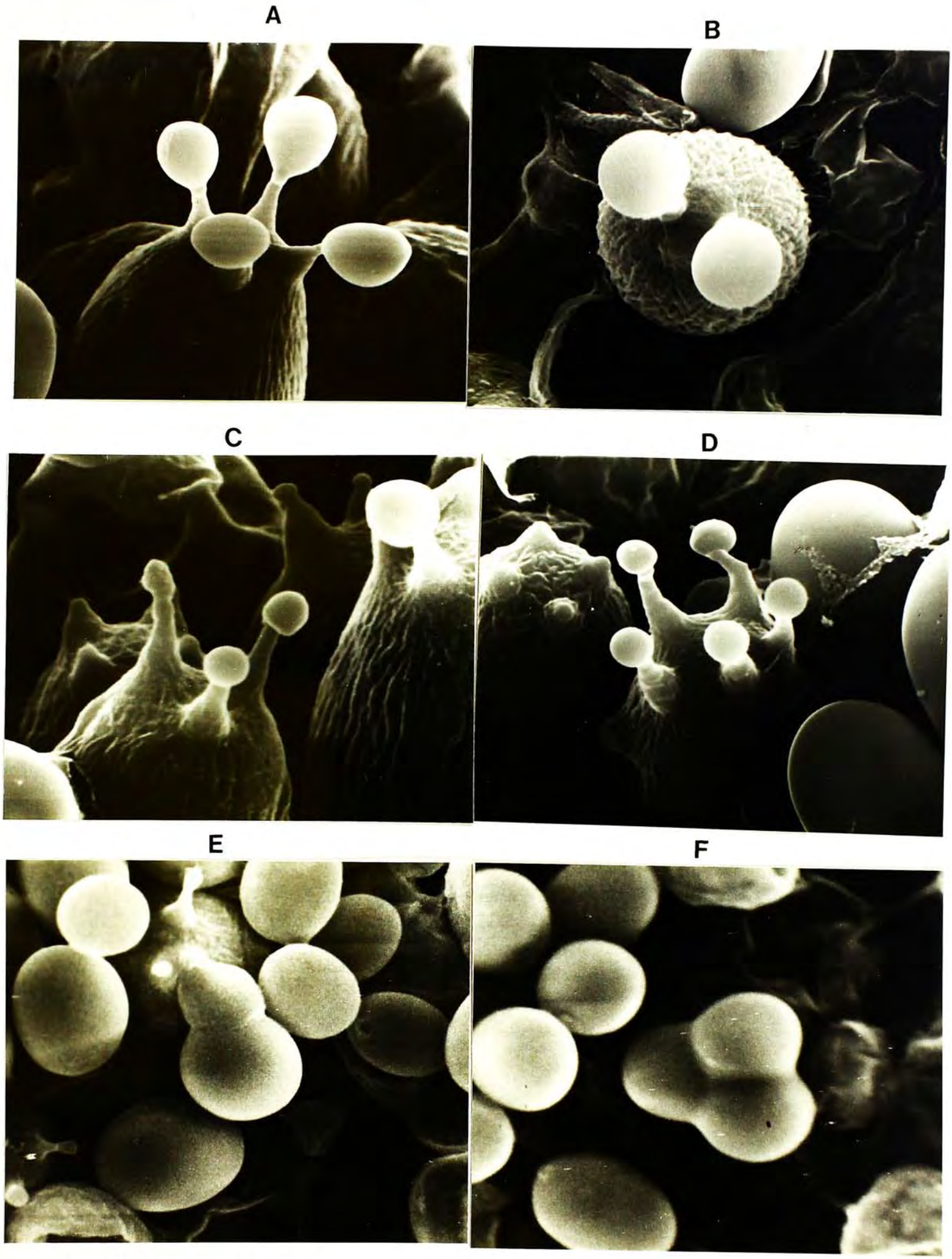


Fig. 5.6. Spore patterns on basidia of strain $V_{42\text{-}18}$. (A). Tetrasporic basidium. (B). Bisporic basidium. (C). Trisporic basidium. (D). Pentasporic basidium. (E). Two spores fused. (F). Three spores fused.

Table 5.4. Distribution of different spore patterns on basidia of *V. volvacea*.

Strains	No. of basidia observed	Bisporic	Trisporic	Tetra-sporic	Penta-sporic
V_{34}	218	4 (1.83%)	6 (2.75%)	206 (94.50%)	2 (0.92%)
V_{35}	224	3 (1.34%)	5 (2.23%)	213 (95.09%)	3 (1.34%)
$V_{42\text{-}18}$	239	4 (1.67%)	11 (4.60%)	221 (92.47%)	3 (1.26%)

5.3.4. DNA content of nuclei in *V. volvacea*

For studying the ploidy of *V. volvacea*, nuclear DNA content of hyphae and basidiospores were measured by fluorescence photometry. Data presented in Table 5.5 shows the fluorescence measurements of nuclear DNA content. It was found that the relative values of nuclear DNA content in basidiospores were similar to that of hyphal cells. It may be considered that both basidiospores and hyphal cells were haploid. Moreover, there was no significant difference between wild type strain $V_{42\text{-}18}$ and self-fertile isolate F-1. However, 22% of the nuclei in self-sterile isolate SN-1 were found to have a lower value of DNA content (>10 a.u.). Distribution of relative nuclear DNA content are shown in the Figs. 5.7-5.10.

Table 5.5. Fluorescence measurements of nuclear DNA content

	No. of nuclei measured	DNA content (a.u.) Mean	Coeff. of Variation (%)
$V_{42\text{-}18}$ (spore)	50	24.166[ab]	28.517
$V_{42\text{-}18}$ (hypha)	50	27.767[a]	28.846
F-1 (hypha)	50	26.888[a]	31.350
SN-1 (hypha)	50	23.150[b]	47.802

$V_{42\text{-}18}$: wild type strain

F-1 : self-fertile isolate derived from strain $V_{42\text{-}18}$

SN-1 : self-sterile isolate with normal morphology derived from strain $V_{42\text{-}18}$

a.u. : arbitrary unit

Values with different alphabets are significantly different at P=0.05 (one way Analysis of Variance followed by Duncan's multiple range test).

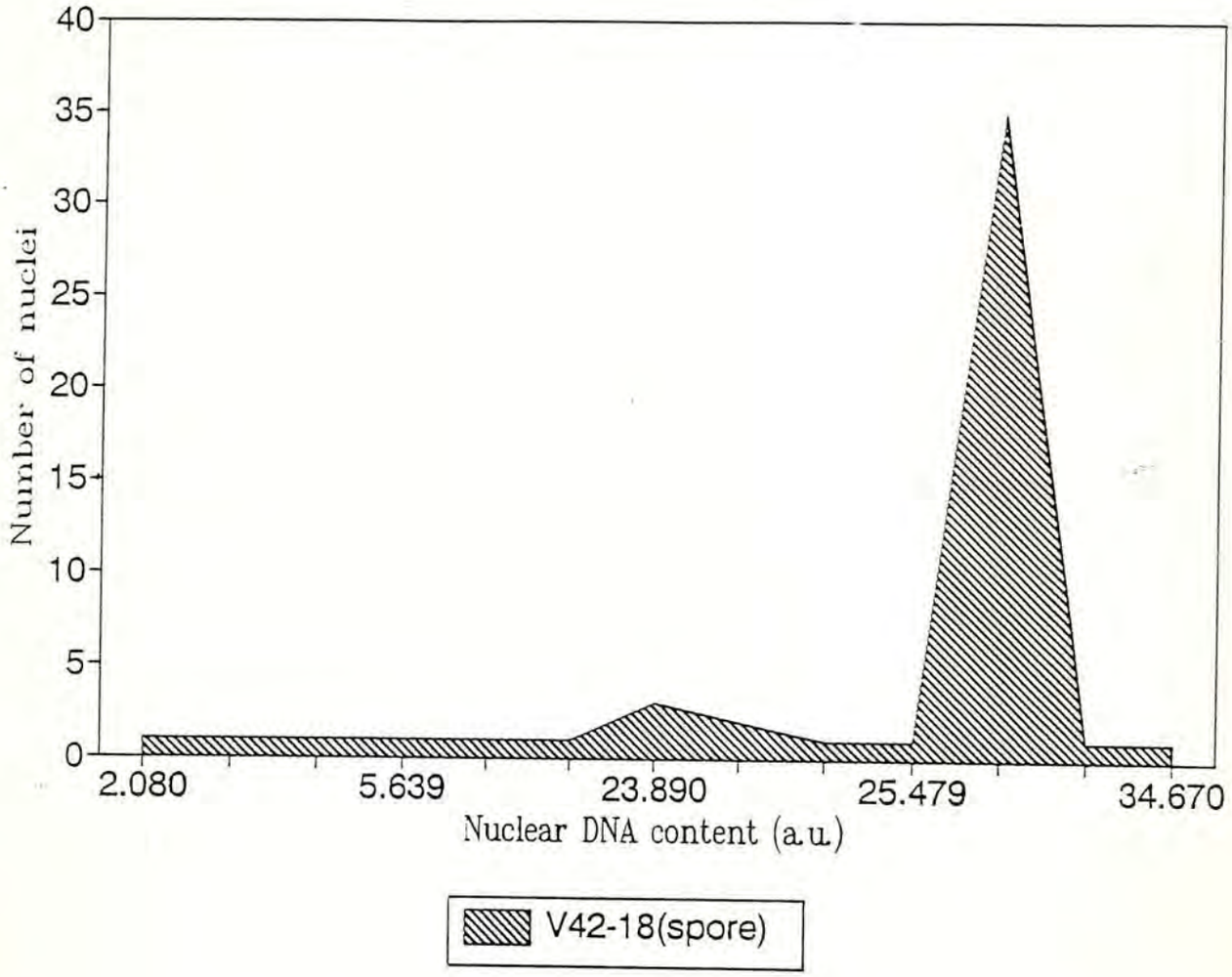


Fig. 5.7. Distribution of relative nuclear DNA content (arbitrary unit) in the basidiospores of strain $V_{42\text{-}18}$.

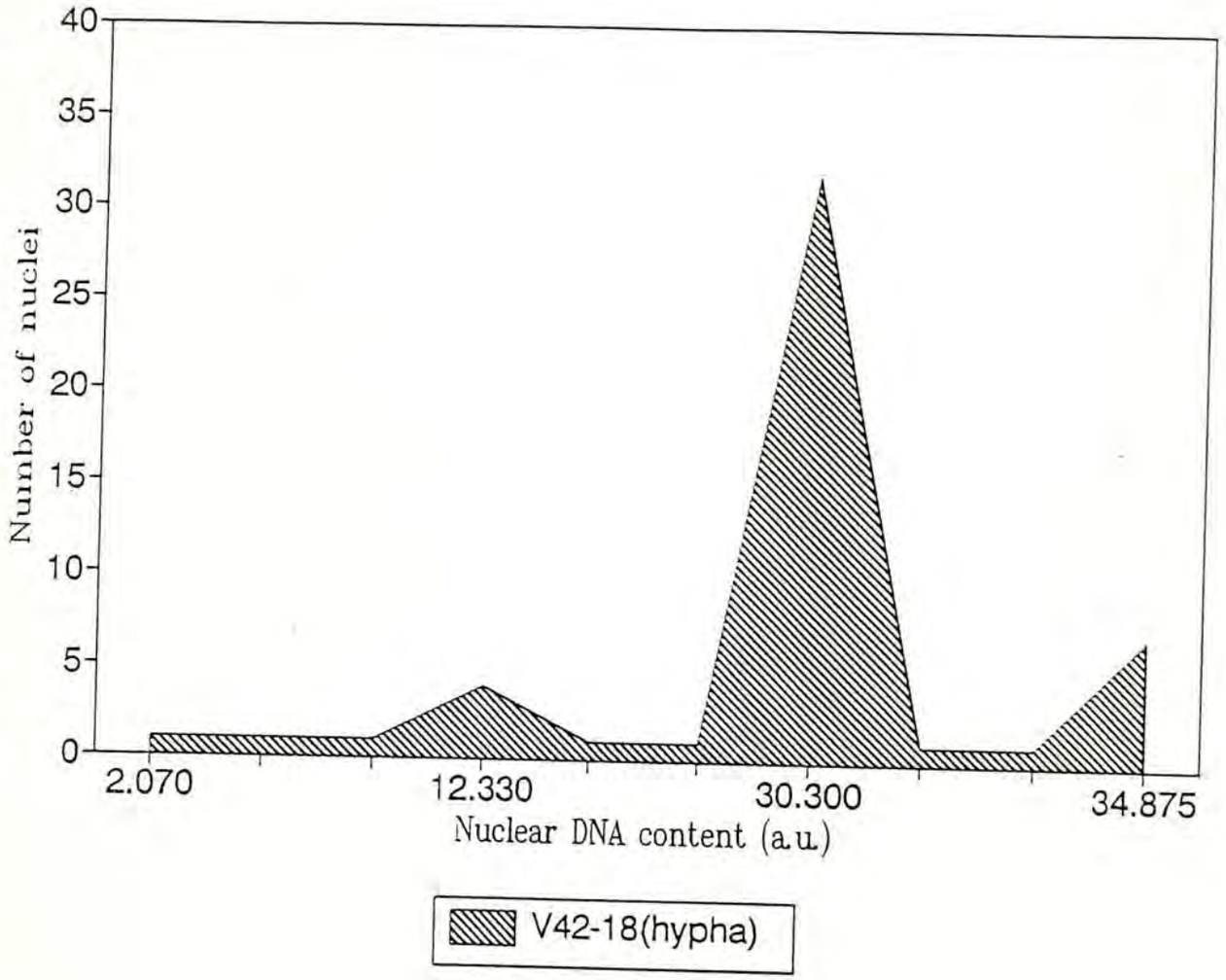


Fig. 5.8. Distribution of relative nuclear DNA content (arbitrary unit) in the hyphal cell of strain $V_{42\text{-}18}$.

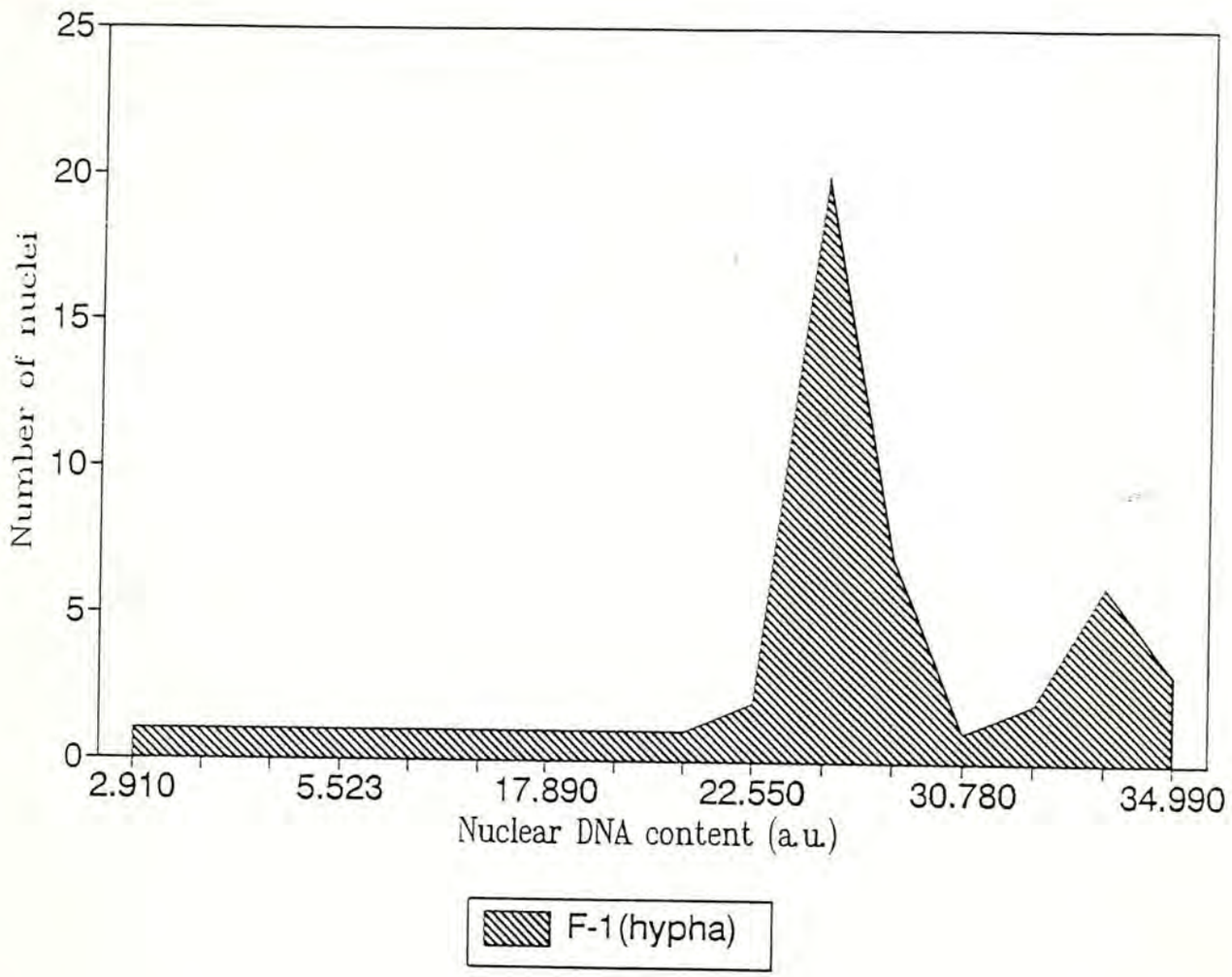


Fig. 5.9. Distribution of relative nuclear DNA content (arbitrary unit) in the hyphal cell of fertile isolate F-1.

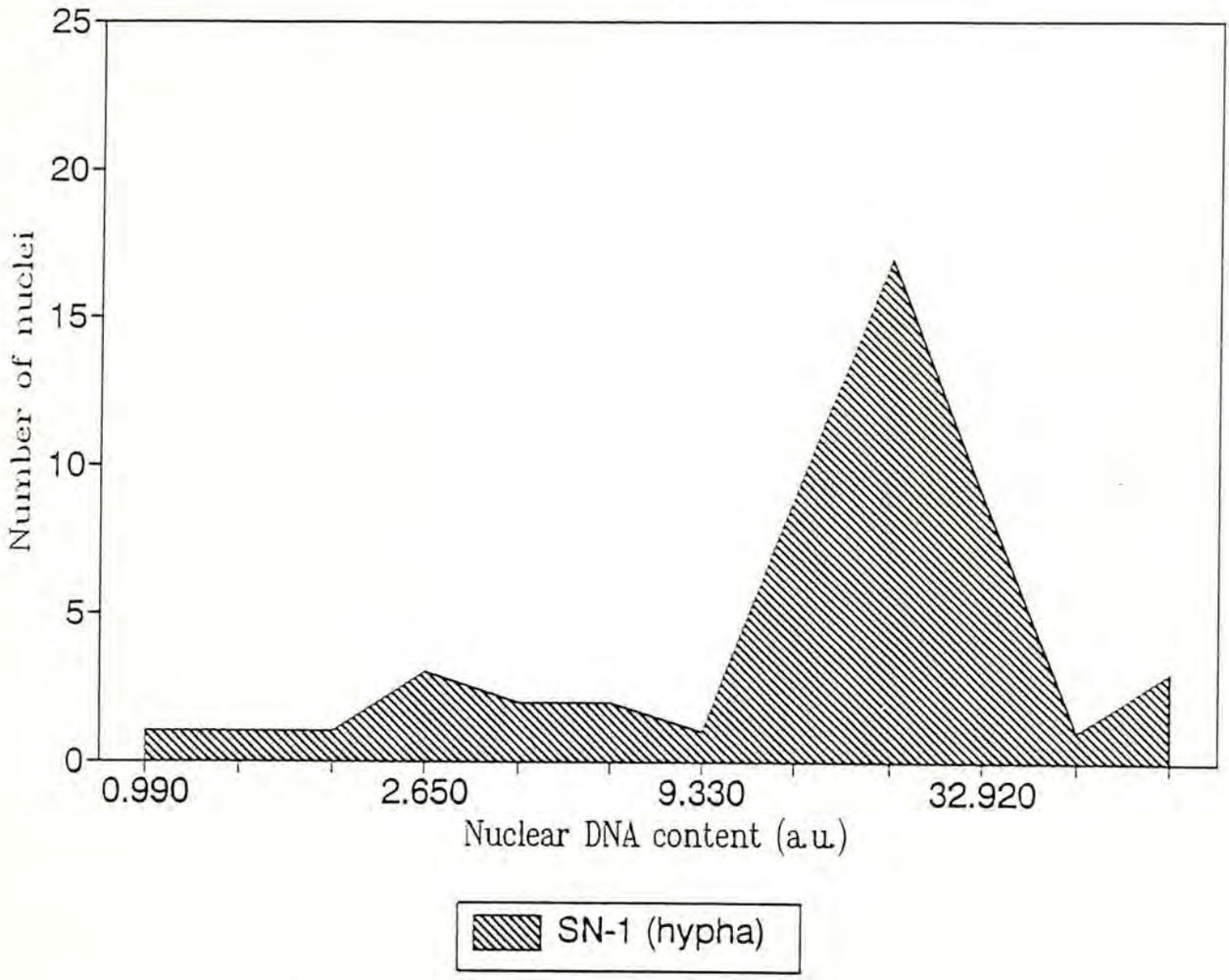


Fig. 5.10. Distribution of relative nuclear DNA content (arbitrary unit) in the hyphal cell of sterile isolate SN-1.

5.4. Discussion

5.4.1. Staining method

For studying the small size nucleus of *V. volvacea*, both Feulgen and DAPI staining methods were used. It is well known that Feulgen reaction is specific to DNA. Mild acid hydrolysis of fixed cells followed by treatment with Schiff's reagent resulted in a discrete magenta colouring at sites of DNA. As pointed out by Barka and Anderson (1965), mild acid hydrolysis splits off the purine bases (adenine and guanine). Removal of the purine bases uncovers potential aldehyde groups, present in furanose form in the D-2-deoxyribose molecules. Exposure of these aldehyde groups to Schiff's reagent then results in the formation of a coloured compound. It is probable that one molecule of Schiff reagent is bound to two adjacent nucleotides.

No satisfactory result was obtained by Feulgen staining method since the images of nuclei were not sharp enough in an unclear background. The fluorochrome DAPI (4',6-diamidino-2-phenylindole) has many attractive properties as a nuclear stain. It binds selectively to AT-rich double-stranded DNA (at least three AT-pairs in a row necessary as binding site); the DAPI-DNA-complex has a much enhanced fluorescence over that of the dye alone and fades comparatively slowly. The fluorescence of DAPI-stained DNA is proportional to the DNA quantity when DNA of the same base composition is

compared (Coleman *et al.*, 1981; Meixner and Bresinsky, 1988; Butt *et al.*, 1989). In the present study, the DAPI staining method was successfully employed in the cytological study of *V. volvacea*. It could produce sharper images of nuclei, with less background than those in Feulgen reagent. On the other hand, this method is also more convenient and more rapid.

5.4.2. Analysis of the possible sources of variation in *V. volvacea*

The number of nuclei in a cell is an important characteristic for cytological study. The results in the observation of DAPI-stained nuclei in hyphae (Table 5.1.) clearly demonstrated that vegetative cells of *V. volvacea* are multinucleate. These results are consistent with those reported by Chang (1969) and Chang and Ling (1970) with Mayer's haematum staining method. In addition, most of the basidiospores were uninucleate, but some contained more than one nucleus (Figs. 5.2 and 5.3). It is worth noticing that the number of nuclei per spore was in the range from 0 to 4.

In addition, more than six hundred basidia have been observed by scanning electron microscope. Although most of mature basidia were tetrasporic, different spore patterns on the basidia were also found (Figs. 5.6, Table 5.4). It The migration of four nuclei after meiosis is very interesting. If the basidium has two, three or five spores instead of four , or if four nuclei mis-migrate into spores after meiosis, the distribution of nuclei in each spore may be different.

Generally, only four nuclei are formed after meiosis in a basidium. Chang (1969) reported that the mature basidia always contain only four nuclei after meiosis. Each of the four spores borne on the basidium does receive one nucleus only. He also observed that the mature spores were commonly uninucleate, but occasionally, binucleate spores were found. Chang assumed the binucleate spores were due to the mitotic division which occurs once more within the spores.

Based on the results of present studies, there are some possible explanations for the variation of nuclear number of basidiospores:

Firstly, one or more of the haploid nuclei in the basidium may undergo mitosis before they migrate into basidiospores. Therefore, more than four nuclei will be present in one basidium. At the same time, unequal distribution of nuclei in basidiospores may occur.

Enucleate spores can be explained on the basis of failure of one of the haploid nuclei to move into the spore. Thus spores having more than one nucleus may result from the movement of one or more meiotic nuclei from the basidium into the spore, but since the number of spores with more than one nucleus greatly exceeds the number of enucleate spores (see Table 5.2), mitotic divisions of the haploid nuclei were more likely to occur.

Another possibility is that multinucleate spores may be the result of

mitosis before the germination of basidiospores. Abnormal meiosis of *V. volvacea* could also be considered.

Li and Chang (1979) proposed several hypotheses to explain the sources of variation for the progeny of *V. volvacea*. For example, "spontaneous mutants", "expression of pre-existing mutants by recombination" or "chromosome aberrations". Since the vegetative hypha of *V. volvacea* is multinucleate, if a mutation occurs in only one nucleus, it may not be detected and the heterokaryotic hypha would have a normal phenotype. However, under certain circumstances, these mutants may get a chance to express their effect (probably through meiotic recombination or an accumulative effect of mutants). This may also lead to a change in phenotype from the normal morphology. Moreover, "non-nuclear factors" may be another source of variation.

With reference to Table 5.3, there is no significant difference in cytological patterns between self-fertile and self-sterile isolates with normal colony morphology. Except the abnormal sterile isolate SA-1, the number of nuclei in wild type strain $V_{42\text{-}18}$ and the isolates, F-1 and SN-1, were also nearly identical. These cytological parameters cannot be used as a indicator to distinguish between fertile and sterile strains.

In 1980 Jackson and Casey (cited from Tolmsoff, 1983) defined heteroploidy as, "...any deviation from the normal chromosome number in a

cell, tissue, or whole organism. Tolmsoff (1983) further qualified the term as follows for application to the fungi: a frequent shifting of chromosome numbers per nucleus, ranging from nullisomics (n-x) through the euploid series of n, 2n, 3n, 4n and so on, and including various aneuploids. Aneuploids play the major role in heteroploidy because most aeuploids of various organisms are unstable. The contribution of aneuploids to variability is also potentially much greater because of the variety of aneuploids that are possible.

Evidence for heteroploidy among fungi has been reported. *Neurospora crassa* has 7 chromosomes in the haploid state. Pittenger (1954) found that pseudo-wild type (PWT) occurred frequently among ascospore progeny in crosses between haploid mutants. He examined 99 isolates of PWTs and concluded that they were disomic aneuploids (n+1) involving five of the seven gene-linkage group. Käfer and Upshall (1973) used genetic markers to identify the specific types of aneuploids of *Aspergillus nidulans*. They found that most of the disomic and trisomic aneuploids were relatively unstable in culture and yielded haploid sectors.

Parry and Cox (1970) used spores from triploid *Saccharomyces cerevisiae* to study aneuploidy. Disomy for individual chromosomes was rare, but disomy for two or more was common, and these often occurred in specific combinations, indicating nonrandom aneuploidy.

Hosford and Gries (1966) found evidence for haploid, diploid, aneuploid,

and polyploid nuclei in the multinucleate hyphal cells of *Phymatotrichum omnivorum*, based upon a "haploid" count of four chromosomes. Molochanova *et al.*(1978, cited from Tolmsoff, 1983) suggested diploidy, polyploidy, and aneuploidy as bases for variability in *Verticillium tricorpus*. They described and illustrated putative spontaneous homozygous diploids and aneuploids based upon unicellular conidial dimensions, nuclear diameters, and interconversions of colony types via single spores with simultaneous changes of conidial dimensions. Michelmore and Ingram (1982) showed evidence that homothallism in the normally heterothallic *Bremia lactucae* is likely due to trisomy for the chromosome carrying mating type or compatibility genes.

Microspectrophotometry of nuclear DNA is playing an increasingly important role in providing quantitative data relative to ploidy level of fungi where chromosome counts are typically difficult and unreliable.

Collins *et al.* (1978) used nuclear DNA measurements and chromosome labelling in genetic studies to demonstrate polyploidy, chromosome loss, and aneuploidy in *Didymium iridis*. Ploidy levels ranging from n to 16n have been observed in *D. iridis* based upon nuclear DNA content. It was also reported that polyploidy is widespread in temperate isolates of *Phytophthora infestans* based on the results of nuclear DNA content, mating type and metalaxyl sensitivity analysis. Tetraploids may be better adapted to the cooler climates, and therefore, favoured by selection pressures (Therrien and Ritch, 1989).

Peabody *et al.* (1978) and Motta *et al.*(1986) did a cytophotometric study of nuclear ploidy (DNA) among different types of cells in *Armillaria mellea*. DNA values are designated as multiples of C, with 1C representing the smallest quantity of DNA observed and assumed to be the haploid (n) state. He found that the majority of *A. mellea* basidiospores were haploid (n=1C), but extended up to 5C. The individual nuclei of dikaryotic hyphae were predominantly 2C (diploid), but extended to 9C. It was also reported that aneuploid oidial colonies occurred from some diploid strains of *Coprinus cinereus* (Murakami, 1989).

Aneuploidy is very common in fungi. There is overwhelming evidence that changes in chromosome numbers per nucleus play an important role in variability between individuals, and in cellular differentiation within an individual.

In the present studies, basidiospores or hyphal cells with lower or higher nuclear DNA content were found in the wild type strain and its monosporous isolates (Fig. 5.7-5.10).However, the order of coefficient variation (c.v.%) is Sn-1 > F-1 > $V_{42\text{-}18}$ > (hypha) > $V_{42\text{-}18}$(spore) (Table 5.5). It means that variation of nuclear DNA content in sterile isolate SN-1 is the largest, but in the spore of strain $V_{42\text{-}18}$, it is lowest. With the reference of Fig. 5.7, about 22% of the nuclei of sterile isolates of Sn-1 have lower DNA content (> 10 a.u.).

Heath (1978) has pointed out that atypical cell division may lead to aneuploidy. It is suggested that variation of nuclear DNA content in *V. volvacea*

may be caused by unequal distribution of genetic materials in daughter nuclei after atypical cell division, such as asynchronous movement of chromosome. As a whole, the data obtained in these studies are highly suggestive of polyploidy or aneuploidy but this can only be confirmed by chromosome counts and more precise methods of measurement. Moreover, pulsed field gel electrophoresis is a new technique to investigate karyotype and chromosomal variation in fungi (Magee, *et al.*, 1988; Orbach *et al.*, 1988; Fox and Peberdy, 1990).

5.5. Summary

1. Both Feulgen and DAPI staining methods were employed in the present study. It was found that DAPI was more convenient and rapid. It could produce sharper images of nuclei with less background than those obtained by using Feulgen reagent. Microautoradiography was also employed to confirm the cytological results.

2. Vegetative cells of *V. volvacea* were found to be multinucleate. The number of nuclei per cell varies widely in the range from two to more than forty, usually fifteen to twenty.

3. Most of the basidiospores were uninucleate but some contained more than one nucleus. Enucleate spores were also observed.

4. A comparative study on cytological differences between self-fertile and self-sterile monosporous isolates was carried out. There were no significant cytological differences between self-fertile and self-sterile isolates with normal colony morphology. However, abnormal sterile isolates had few hyphal branches with compact morphology.

5. Results from scanning electron microscopic examination on the hymenium of three strains show that most of the mature spore patterns on basidia were tetrasporic. However, different spore patterns on basidia were also

observed. The number of spore on a basidium varies from two to five.

6. Based on the results from the study of nuclear DNA content by means of microphotometer, aneuploids were found in the wild type strain and its monosporous isolates, especially in the abnormal self-sterile isolates.

7. The possible sources of variations of *V. volvacea* were also discussed.

Chapter Six: General summary and conclusion

Although *Volvariella volvacea* has been cultivated for many years, many basic genetical and cytological characteristics of this edible mushroom are still not fully understood. About twenty years ago, great variations in monosporous isolates were first reported by Chang and Yau (1971) and confirmed by Li and Chang (1979) and Chang *et al.*(1981). It is very important to investigate the sexuality and mechanism of variation of *V. volvacea*. Academically, it could explain the nature of homothallism on which there are many arguments and interpretations. On the other hand, the genetical exploration of possible sources of variation could also contribute to the development of some meaningful programmes for strain improvement in the straw mushroom.

This thesis can be broadly divided into three parts:

The first part involved to phenotype variation studies. Totally, 328 monosporous isolates derived from five geographical strains of *V. volvacea* were used to analyze their characteristics. In addition, a special device for fruiting was set up to investigate their self-fertility.

Secondly, a genetical approach was conducted in an attempt to discover of the problem why great variation occurred in this homothallic species. To achieve this goal, attempts have been made to induce auxotrophic or resistant mutants with physical and chemical mutagenesis as well as enrichment

procedures for selection of auxotroph. Moreover, the progeny analysis of mutants was also performed.

Third, cytological studies on the nuclear behaviour and differences between self-fertile and self-sterile isolates of *V. volvacea* were carried out. The spore pattern on basidia was examined by means of a scanning electron microscope. In addition, nuclear DNA contents were measured by using microphotometer to see whether any aneuploids were formed in the straw mushroom.

The experimental results showed that there was a wide range of variation in monosporous isolates even though they were derived from one sporocarp. These variations occurred not only in growth rate but also in colonial morphology, fertility, and cellulase activity. Self-sterile isolates were usually found to be medium or slow growers. However, isolates with fast growth rate could be self-fertile of self-sterile. Production of chlamydospores was not related to the colonial morphology.

In the present study, induction of mutants in *V. volvacea* was very difficult due to the multinucleate nature of hyphal cell. If basidiospores were used to induce mutant, their germination rate was very low. On the other hand, instability and sterility of mutants were the main problems hindering the analysis of mutant progeny. Totally, 199 putative resistance mutants were obtained, only 4 had stable phenotype and were also fertile. Results from progeny analysis of

mutant strains *asp*⁻ and *VmgR*, *V14mgR1* showed that over 90% progeny retained their parental mutant phenotype. It could be concluded that *V. volvacea* is a primary homothallic species. Moreover, by analyzing the crossed cultures of different mutants with various markers, heterokaryosis is found although clamp connections are absent in this species.

Results from cytological studies demonstrated that there were no significant differences between self-fertile and normal morphologically self-sterile isolates, whereas, abnormal sterile isolate had few hyphal branches with compact pattern of mycelia.

Although most of the basidiospores were uninucleate, the occurrence of binucleate spores and multinucleate, even non-nucleate spores may be caused by mis-migration of meiotic nuclei from basidium to spores. Moreover, different spore patterns including not only tetraspores but also bispores, trispores and pentaspores were observed in basidia under the scanning microscope. In addition, aneuploids with lower or higher nuclear DNA content when they were compared with the mean value were found based on the measurements with microphotometer. All these genetic material or nuclei unequal distribution in basidiospores could account for the phenotypic variation in *V. volvacea*.

Suggestions for further works are listed below:

1. Stable and fertile mutants are very important in this project study. To

obtain more mutants, protoplast technique is worth considering since protoplast is more sensitive to mutagens than intact cells. Although it was reported by Mukherjee and Sengupta (1986) that no morphological or auxotrophic mutants were obtained from protoplasts by chemical mutagenesis in *V. volvacea*, this attempt is worth trying. A study is already under progress in our laboratory.

2. Restriction fragment length polymorphisms (RFLPs) have been successfully used as unambiguous genetic markers in some mushrooms (Castle *et al.*,1987; 1988; Horgen and Anderson, 1989; Summerbell *et al.*,1989).If DNAs from homokaryotic isolates have less complex patterns than heterokaryotic strains in Southern DNA-DNA hybridizations, then this characteristic could be used to confirm the recovery of homokaryons from heterokaryons. Crosses between homokaryons could be a rapid method of forming strains with novel genotypes. On the other hand, the polymerase chain reaction (PCR) technique, invented in 1985, can produce large quantities of specific DNA from small, degraded, and impure samples (Oste, 1988; Gyllensten, 1989; Williams, 1989; Arnheim *et al.*,1990; Bloom,1990). It was reported that PCR amplification of rDNA sequences from a single spore of *Neurospora tetrasperma* was possible (Lee and Taylor, 1990).

Recently, a new method, Arbitrarily Primed Polymerase Chain Reaction (AP-PCR) was developed (Welsh and McClelland, 1990; Welsh, *et al.*, 1991; Williams, 1989). AP-PCR does not require a particular set of primers. Instead,

this method uses primers chosen without regard to the sequence of the genome to be fingerprinted. Each primer gives a different pattern of AP-PCR products. Polymorphisms in genomic fingerprints generated by AP-PCR can distinguish between strains of almost any organism. By using these methods, it could be identified whether the sporocarp is produced from a monosporous isolate for the study of sexuality pattern of *V. volvacea*.

3. Microphotometry is becoming an indispensable tool for quantitative analysis of microscopic images. Variation of nuclear DNA content in *V. volvacea* was found in the present study. However, the formation of aneuploids and the relationship between aneuploid and variation remained to be further investigated. In addition, the results can also be confirmed by study of the electrophoretic karyotype.

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